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THE ISOLATION AND STUDY OF NITRIFYING BACTERIA[1]

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INTRODUCTION

The formation of nitrates from organic nitrogenous substances in soil has been known for a long time. As far back as the middle of the nineteenth century the nature of this process was discussed and was considered by the majority of investigators to be purely chemical. This chemical view was held by Kuhlman (30), Dumas (17), Mulder (36) and other investigators, and was further substantiated by the great chemist Liebig (32) whose powerful influence no doubt prevented an earlier discovery of the actual cause of nitrification. The results of Boussingault (12) (1860) demonstrated that the nitrogen of nitrates does not come from the supply of the air. In 1878 Schlöessing and Müntz (51), from a study of sewage disposal, presented a most remarkable paper in which they demonstrated that the oxidation of ammonia to nitrite is due to microörganisms found in soil. Their results led to numerous attempts to isolate the organism, but without success. Warington (59, 60, 61), Frank (18), Frankland (19), Adametz (1), and Heräus (24) attempted to secure the organisms in pure culture but were unsuccessful.

HISTORICAL REVIEW

Warington (59, 60, 61) began the study of nitrification prior to 1878, and continued it through to 1890. Although his cultures contained a large number of ammonia-oxidizing organisms, he was not able to isolate on agar or gelatin-plates pure cultures of this organism. He secured many pure cultures from plates but never the true ammonia oxidizer. Similar attempts to secure pure cultures of the nitrite-oxidizing organism resulted in failure.

The Franklands (19) (1890) worked on the problem of the isolation of the nitrite-forming organism about the same time as Warington. In 1890 they published a report in which they state that all contaminating forms were eliminated from their cultures, except one, which would grow in bouillon but would not oxidize ammonia. By repeated dilution experiments they claim to have

eliminated this last form and thus to have secured a pure culture of the nitrite-forming organism.

Jordan and Richards (27) (1888–1890), observed the oxidation of ammonia to nitrite and nitrate in samples of natural waters. Upon investigation they found that the nitrifying organisms were invariably present in Boston tap-water. Attempts to isolate the organism by the gelatin plate method resulted in failure. Many organisms were isolated but none would oxidize ammonia in pure culture or with a mixed culture of strains isolated from the original water. Dilution methods were employed and an organism obtained which oxidized ammonia to nitrite and subsequently to nitrate. Jordan and Richards are the first American investigators to report the isolation of nitrifying bacteria.

The classical nitrification experiments of Winogradsky (63, 64, 65) beginning in 1890, stand out preeminently, and have never been repeated. The results of his earlier work, namely, the discovery that organic matter in culture media is toxic to sulfur bacteria, no doubt proved very helpful in his investigation of the nitrifying bacteria. Winogradsky was able to isolate the nitrifying organisms by use of a medium containing strictly pure inorganic salts. First he carried his mixed cultures through many enrichments, i.e., several transfers in selective media. He then inoculated gelatin plates from these enrichment cultures and secured the ammonia-oxidizing organism (*Nitrosomonas*) in pure culture by the negative plate method, i.e., from portions of the plate which showed no visible growth. Later silicic acid gel plates were prepared, containing ammonium sulfate and other inorganic salts, and the colony characteristics of the organism studied in pure culture. Washed agar was used with similar success. One year later he repeated the work and isolated the nitrite oxidizer (*Nitrobacter*). Winogradsky states that both nitrifying organisms are slow to develop on solid media and are visible first under the microscope after an incubation period of 2 to 6 weeks. Growth on the plates is detected first by chemical tests. After all of the ammonium sulfate or the sodium nitrite is oxidized more is added in small wells made by removing portions of the medium, and in this manner the size of the colonies increased. He describes the nitrite-forming organism (*Nitrosomonas*) as an oval or ellipsoidal cell 0.9 to 1 μ wide and 1.2 to 1.8 μ in length. There are two types, one persisting in zooglea-like growth, the other in single cells; the former produces no turbidity in the solution, while the latter produces turbidity. The single-celled form, termed "Schwärmer" is motile, with a single polar flagellum. The colonies on silicic acid gel appear (enlargement of 100 diameters) as small refractive bodies with sharp outline, at first colorless, later brown and finally a characteristic dark brown. Some colonies remain compact while others have a main central kernel surrounded by single cells. The colonies are exceedingly tenacious and difficult to tear apart with the platinum needle. The nitrate-forming organism (*Nitrobacter*) is 0.3 to 0.4 μ wide and about 1 μ long, the cells occurring singly or in pairs and occasionally in threes. They

are spindle-shaped, non-motile, and possess a capsule which makes them difficult to stain. After a period of 10 days colonies on washed agar appear as strongly refractive bodies at a magnification of 150 to 200 diameters, and after two to three weeks are rounded, oval, or heart-shaped, 30 to 50 μ in diameter, somewhat brown and shiny. Winogradsky isolated these organisms from the soils of many different countries and found that they varied only slightly in morphology.

He carried out many experiments and found in all cases that soluble organic matter is very toxic to these organisms. Moreover, in coöperation with Omeliansky (67), he noted that glucose, peptone, asparagin, glycerin, and urea are fatal in quantities of 0.2 to 1 per cent. Bouillon was used as a test for purity, and if no growth was visible at the end of 10 days the culture was considered pure. They found that the carbon must be derived from free carbon dioxide or the bicarbonates; carbon from organic substances or from normal carbonates is not available. For each part of carbon assimilated by *Nitrosomonas* 35 parts of nitrogen are oxidized to nitrite, and for each part assimilated by *Nitrobacter* 40 parts of nitrite nitrogen are oxidized to nitrate.

From 1892 to 1896, Godlewski (21, 22, 23) worked with nitrifying organisms and by means of a series of carefully planned experiments confirmed the work of Winogradsky in regard to their source of carbon. He later repeated the work in mixed cultures and found that nitrification will not take place unless carbon dioxide is present.

In 1895 Burri and Stutzer (13, 14) claim to have isolated the nitrate-forming organism, and stated that it grew in bouillon. After a controversy of considerable duration, Winogradsky (65) secured one of their supposedly pure cultures and found that it contained other organisms. He isolated from this culture the true nitrate-forming organism and two other forms. Two years later Stutzer and Hartleb (56) took up the problem again, made enrichment cultures, and inoculated agar plates. After a period of 4 to 6 weeks the nitrite in the plates was oxidized and colonies developed which converted nitrite to nitrate in liquid media but would produce no growth in bouillon. Agar slopes containing nitrite were inoculated and after a long period of incubation growth developed which was not continuous over the agar but made up of many very small beads. When examined in the hanging drop, the organisms were found to have yeast-like buds. They also isolated *Nitrosomonas* by use of magnesium-ammonium-phosphate ($MgNH_4PO_4$) agar plates. On this phosphate medium colonies developed after 4 weeks; but no growth could be secured on silicic acid gel. Stutzer claims to have isolated the organisms from the gel by the negative method.

In 1897 Stutzer and Hartleb (56) attempted to explain the presence of the contaminating forms found in their culture by Winogradsky, and did further work on the carbon relation of the organism in pure culture. Four years later they published a remarkable paper (57) concerning the isolation of the nitrifying organisms. By the use of enrichment cultures and washed agar they isolated

a nitrate-forming organism to which they gave the name *Nitromicrobium*. Their description of the physiological properties of the organism is similar to that given by Winogradsky for *Nitrobacter*, and in morphology it varied but little from *Nitrobacter*, the chief difference being a yeast-like budding of *Nitromicrobium*. They reported also the presence of another organism which was strikingly similar to *Nitrobacter* and *Nitromicrobium* but did not oxidize nitrite to nitrate. This non-nitrifying form they called *Hyphomicrobium*. The colony characteristics were much the same as those of *Nitrobacter*. In common with *Nitrobacter*, a medium containing nitrite was essential for the growth of *Hyphomicrobium*. This organism was oval in shape, 0.8 μ wide and 1.2 to 1.5 μ long. It would not grow in bouillon but would grow in a solution containing asparagin or ammonium sulfate. The greatest difference between *Hyphomicrobium* and *Nitrobacter* was a thread-like growth of very small rods which could be seen growing from one pole, or sometimes both poles, of the *Hyphomicrobium* cells. The authors are firm in their statement that Winogradsky did not at all times have a pure culture because he used the bouillon test as a criterion of purity and there are other organisms which will not grow in bouillon, for example *Hyphomicrobium*. This report contains 21 photomicrographs of the organisms. Stutzer (55) later reported on the isolation of *Nitrobacter* by means of unwashed agar plates. The bud-like growth was again observed. Magnesium-ammonium-phosphate agar plates were used successfully for the isolation of *Nitrosomonas*. Silicic acid gel was also used but did not give satisfactory results. The nitrite-forming organism was secured from the gel by the negative method.

In the literature on nitrification it seems that this report of Stutzer and Hartleb has been overlooked. The writer of the paper presented herewith has noticed a very short stem-like growth in many stains of his pure cultures of *Nitrobacter*. Such growth is very noticeable when the preparation is stained by any method for staining flagella, but is seldom seen in the ordinary stained preparation. Results obtained by the writer lead him to believe that these investigators were nearer the truth than is commonly thought.

Beddies (5, 6), in 1899, reported the isolation of nitrifying organisms. He found that the organisms were not very sensitive to high concentrations of organic matter and were aided by the presence of compost and humus in the medium. The solid medium he used consisted of silicic acid gel containing 1 per cent of a concentrated humus solution. The organisms were able to form spores under certain conditions.

In 1899, Omeliansky (40–44) isolated the nitrite-forming organism and introduced gypsum blocks as a suitable solid substratum. He prepared them by adding 1 per cent magnesium carbonate ($MgCO_3$) to calcium sulfate ($CaSO_4.H_2O$) and mixing with sufficient water to make a dough, which was then spread out on a smooth surface and allowed to dry. When hard the blocks were placed in petri dishes, sterilized, and the solution containing the desired salts poured into the dish. The nutrient solution readily diffused through

the solid gypsum block, the surface of which was inoculated with the desired culture. He reported a yellow, wart-like growth on the surface of the block. This method has the advantage that the old solution may be removed and fresh solution added; it has the disadvantage that observation under the microscope is difficult and unsatisfactory. In later reports (45, 46) Omeliansky used filter paper pads instead of gypsum. He found *Nitrosomonas* was easily stained with carbol fuchsin, but that *Nitrobacter* was much more difficult to stain.

Boullanger and Massol (11) (1903) claim to have isolated both of the nitrifying organisms. They state that silicic acid gel was used as the solid medium but do not give any cultural characteristics or purity tests. They found the thermal death point of *Nitrosomonas* to be 45°C., and that of *Nitrobacter* 55°C. A concentration of 30 gm. of ammonium sulfate in 1 liter greatly retarded nitrite formation and 20 gm. of magnesium nitrite ($MgNO_2$) retarded nitrate formation.

Fremlin (20) (1903) reported on the isolation of *Nitrosomonas*. He secured enrichment cultures and inoculated plates of silicic acid gel, agar and gelatin. Growth developed on all the plates. He claims to have obtained a pure culture. He reported that *Nitrosomonas* would grow in organic media. This conclusion was further substantiated by inoculating a slope of beef-broth agar from the pure culture, and securing a moderate growth. A nutrient ammonium sulfate solution was inoculated from the slope, and the solution then filtered several times through sterile soil under aseptic conditions. The ammonia was oxidized to nitrite while a control solution filtered through soil in similar manner produced no oxidation.

Berstyn (8) (1903) describes with exceptional thoroughness the forms which contaminate *Nitrobacter* enrichment cultures. He found the following species in the F_{20} generation of *Nitrobacter* cultures,—*Bacterium comes, Bacterium modestum, Bacterium debile*, and *Pseudomonas humicola*. He states that these forms are easily satisfied in food requirement and develop in bouillon or gelatin in very dilute solution, but will not remain alive in distilled water. They develop in the inorganic nitrifying solution unless the salts used have been recrystallized and the water distilled from permanganate and sulfuric acid. If this precaution is taken the forms will be eliminated in the enrichment process. A very insignificant amount of organic matter is necessary to meet their requirement.

Wimmer (62) (1904) claims to have isolated the organisms, although his methods are not given. He states that at first neither *Nitrosomonas* nor *Nitrobacter* grew in bouillon, but in later experiments with the same cultures *Nitrobacter* grew. From tests with the cultures made in sand, the author concludes that organic matter is not so toxic as in solution, but is nevertheless toxic in great concentration.

Perotti (48, 49) (1906) experimented with the nitrite-forming organism. These were found in groups of 2 to 6 collected on the magnesium carbonate in

the bottom of the flask, but no zooglea forms were noted. He considered it the same as the organism described by Winogradsky, the "Westeuropaischen" form.

Bazarewski (4) (1906) isolated pure cultures of the nitrate-forming organism. Experiments were carried out in sand, the results of which confirmed other reports concerning the toxicity of soluble organic compounds. On the other hand, organic matter in soil was found not toxic. The most favorable temperature in pure culture was 37°C. In soil and sand cultures 1 per cent of dextrose stimulated nitrate formation while larger amounts delayed the process but did not entirely prevent it. From his results the author concluded that denitrification and nitrification go hand in hand in soil.

Coleman (15) (1908) worked with the nitrifying organisms and performed experiments in sand and soil. He found that 1 per cent of dextrose was not toxic, but that 2 per cent was extremely toxic. Tests for purity were made with bouillon and Heyden Nährstoff agar. The culture of *Nitrosomonas* was reported impure. The ratio of nitrogen to carbon for *Nitrobacter* was found to be about 40 to 1.

Owen (47) (1908) reported the isolation of pure cultures of the nitrifying organisms and experiments with pure cultures. The organisms were used in the study of "The Effects of Carbonates on Nitrification." Very little detail of the work of isolation is reported. He states that plates were made on washed agar and pure cultures secured from the colonies which developed. The agar gave better results than silicic acid gel. No specific tests for purity were made. Photomicrographs of the organisms show vast differences in size and shape of the individual cells.

Makrinoff (34) (1909) isolated *Nitrosomonas* and *Nitrobacter* and carried out experiments to test the effect of organic matter on pure cultures. He prepared gypsum plates (10 gm. $CaSO_4.H_2O$) containing from 0.25 to 6.5 gm. of soil each, and after the addition of the nutrient solutions inoculated them with pure cultures of the organisms. The soil did not prove toxic but caused a darker color in the organisms on the plates containing the higher amounts. Quantities of soil from 0.5 to 12.5 gm. were placed in 20-cc. portions of liquid medium, and then sterilized. The cultures receiving the 12.5 gm. of soil required 20 days for nitrite formation, while those receiving the smaller amounts of soil required only 5 days. Soil extract stronger than 2 per cent inhibited oxidation. However, a solution containing 2 per cent of soil extract was not toxic when used with plates of gypsum. The report contains photomicrographs of the plates, showing the growth of the organisms, which in general was abundant. This is an important paper on the subject of nitrification.

Beijerinck (7) (1914), reported on the isolation and cultivation of *Nitrobacter* on silicic acid gel and agar plates. He found the organism much the same in every respect as that described by Winogradsky, except in its relation to bouillon. His culture of *Nitrobacter* grew in bouillon; but by so doing it lost its power to oxidize and did not regain this power of oxidation even after

the lapse of 10 years. The oxidizing form he termed *Nitrobacter oligotrophic* and the non-oxidizing form in bouillon he termed *Nitrobacter polytrophic*. The crude cultures contained several contaminating forms which were very difficult to separate from the nitrate-forming organism. Several of these forms belonged to the family of *Actinomycetes*. The chief forms found in the nitrifying cultures he termed *Actinobacilli*. A small bacillus was also described which formed a dark brown or red pigment and was similar in gross appearance to the nitrate-forming organism. The red pigment was identified as carotin. *Bacillus nitroxus* was also present and proved the most difficult of all to separate from *Nitrobacter*. The development of unusually large colonies in pure culture on agar indicated the transformation from the active to the inactive stage; the large colonies never again produced oxidation when transferred to suitable media. He found that less than 1 per cent of sugar, mannite, sodium or calcium acetate, peptone, tyrosin, or asparagin caused the loss of oxidizing power. Colonies were evident on washed agar within 2 days. The fact is strongly emphasized that pure cultures do not grow so rapidly as mixed cultures.

Joshi (28) (1915) reported a new nitrite-forming organism which showed decided pleomorphism. This organism was commonly found in two forms, one of which was chalky white in appearance, thread-like, and long and branching like a mold, while the other form was shorter and had flagella at one pole. It would not grow in bouillon or on gelatin, and preferred magnesium carbonate as a base. The thermal death-point was found to be between 70 and 80°C. His work has not been confirmed by any other investigator.

Allen and Bonazzi (2) (1915) began the enrichment process preliminary to the isolation of the organisms. The *Nitrosomonas* cultures were carried to the F_4 generation but were abandoned at that point because of their low oxidizing efficiency. *Nitrobacter* was carried to the F_{18} generation with success. Neither organism was isolated. Bonazzi (9) in 1919 published a report which deals with the conditions favorable for nitrite formation. He found that a slow rotary movement of the culture greatly increases oxidation.

In his first experiment he states that the solution was inoculated with a pure culture. In experiments immediately following, solutions were inoculated with cultures nearly pure, while in later experiments the solutions were inoculated without reference to purity. It is apparent throughout the work that the author has met with difficulty in experimentation with pure cultures. He later reported (10) on the isolation of the nitrite ferment and gave a brief description of the organism in pure culture. There can be no doubt that the author actually obtained a pure culture of *Nitrosomonas*. This latter report is very brief and the methods employed are inadequately described. The tendency of the organism to lose its oxidizing power as a result of cultivation in liquid medium is admitted, and the necessity for special apparatus to insure aeration emphasized.

Hopkins and Whiting (25) (1916) reported on the isolation of *Nitrosomonas*. This report is indeed unusual. The details of isolation are not given; but experiments with pure cultures are discussed. In liquid cultures colonies formed on the surface in a bluish mass, some developing to $\frac{1}{4}$ inch in diameter. Silicic acid gel, inoculated from impure soil infusion, developed colonies of *Nitrosomonas* $\frac{1}{8}$ inch in diameter. They were colorless to opalescent at first and later a glassy blue, with the center showing yellow after 14 days and later orange-yellow to brown. "Visible growth in solution was slow for the first 40 days; but after that time a very profuse surface growth developed showing large blue colonies some of which were drawn up the sides of the flask by the surface tension of the liquid, and there developed to a large size ($\frac{1}{4}$ inch in diameter)." They state that *Nitrosomonas* rendered rock phosphate soluble in artificial medium and therefore acted similarly in the field. This conclusion provoked considerable criticism. The writer has made repeated attempts to confirm this work of isolation, but has never been successful. No other investigator has recorded the development of such large colonies even after extensive investigation; in fact the persistently small colony is a confirmed characteristic of both nitrifying organisms.

Russel and Bartow (50) (1916) reported work with pure cultures of *Nitrosomonas* and *Nitrobacter* isolated from activated sludge. Washed agar and silicic acid gel prepared after the method of Stephens and Temple (53) were used in isolation. Both types of media afforded visible growth after an incubation of 9 to 11 days, but no growth could be secured on gypsum blocks. The organisms would not appreciably oxidize the sludge after they had been isolated, but required the addition of a portion of fresh sludge or mixed cultures of organisms isolated from the sludge. The authors consider this proof that the nitrifying bacteria require the aid of other forms to oxidize the sludge in a sewage disposal plant. However, they state that *Nitrosomonas* and *Nitrobacter* are responsible for the nitrification of the sludge. Very little proof for the purity of their cultures is presented.

Müntz (37, 38) (1890) reported on the occurrence of nitrifying organisms. They were found in great numbers at the summit of high rocky cliffs, where nothing was present but bare rock, from which he concludes that the organisms are important in the decomposition of rock. The period of their development in this particular place was very short because of the relatively short summer. He also reported their presence in ice-clad material which had been in that condition for many years, the organisms remaining alive but inactive.

Jensen (26) (1899) studied the presence of the nitrifying organisms in the soils of Denmark. His determinations consisted of inoculating ammonium sulfate nutrient solutions with small amounts of the various soils. In peat and highland soils alike, he reported the presence of very small numbers of these organisms. Experiments with old soils of various types also indicated the presence of nitrifying organisms in very small numbers.
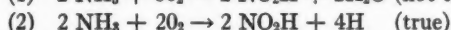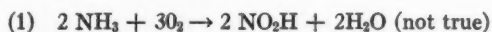
Thomsen (58) (1907) reported the presence of nitrifying bacteria in the sea. He found the organisms in the water at all depths up to 100 meters and often at even greater depths. However, they were found only in water adjacent the shore which indicated that the organisms were recently carried out by the water currents.

Karpinski and Niklewski (29) (1908) experimented with the nitrifying organisms in crude cultures, "Rohkulturen," and confirmed the conclusions of other investigators in regard to the toxic action of soluble organic matter. Niklewski (39) later made extensive experiments to determine the presence of these organisms in manure and urine. From his experiments he concluded that they were present in fresh manure and urine but always from outside contamination. They were found in numbers as high as 10,000 per gram in the outer layers of relatively fresh manure, while the deep manure contained only a very small number, probably because of insufficient aeration. Manure extract and urea exerted a toxic action on these organisms. The organisms found were considered to be the same as those isolated by Winogradsky.

Millard (35) (1911) reported on the number of nitrifying organisms found in soil as determined by the selective culture method. He observed a maximum number of 100,000 nitrifiers per gram of dry soil. No nitrifying bacteria were found in cow dung.

Leone and Magnanimi (31) (1891) reported on the spontaneous nitrification of gelatin in solution. After 45 days at 32°C. the nitrogen was converted to nitrate. They found more nitrogen at the end than at the beginning of the experiment, indicating the chemical fixation of nitrogen from the air.

Loew (33) (1891) discussed the following equations for the oxidation of ammonia to nitrite in the nitrification process:

$$\text{(1)} \quad 2 NH_3 + 3O_2 \rightarrow 2 NO_2H + 2H_2O \text{ (not true)}$$
$$\text{(2)} \quad 2 NH_3 + 2O_2 \rightarrow 2 NO_2H + 4H \quad \text{(true)}$$

He holds that equation (1) is not true but that equation (2) represents true conditions. The hydrogen is used to reduce the carbon dioxide as follows:

$$CO_2 + 4H \rightarrow CH_2O + H_2O$$
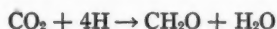
The formaldehyde (HCHO) is not condensed to a carbohydrate but is directly synthesized to protein.

PURPOSE OF INVESTIGATION

This is a report of investigations planned to study primarily the relations of the nitrifying bacteria to their environment. It is reasonable to believe, if these organisms can be isolated and can be brought to function in pure culture, that some of their relations may be explained. If they cannot be made to function normally in pure culture, then the result is equally important. Nitrification will not take place in a solution which is acid, yet some acid soils

support nitrification even better than soils which are neutral or alkaline in reaction. Whether or not this is true with pure cultures has never been determined. It is possible that other organisms present, which are favored by the acid condition of the soil, make possible the growth of the nitrifying organisms. If such a symbiotic relation exists, it can be determined only by the use of pure cultures. The fact that organic matter is not toxic in soil when mixed cultures are used does not signify that such matter is not toxic to pure cultures. The breaking down of this material through the action of other organisms is generally accepted as the explanation of the fact that soil organic matter is not toxic; yet this fact has never been established by experimental evidence. Work with pure cultures might determine whether or not this hypothesis is correct.

In addition to the many questions which present themselves, the problem of securing pure cultures is a large one in itself. The methods of isolation have been improved but little since the appearance of Winogradsky's first paper on this subject. The relationship which exists between these organisms and their related forms is still a mystery. The bouillon test for purity has given conflicting results and we do not know whether or not this can be used as a test for purity. This paper deals with the work of isolation and morphology of the organisms, a presentation of methods employed and results secured. It is hoped at a later date to publish a more complete report of the physiology of these organisms in pure culture.

EXPERIMENTAL DATA

Methods

The methods and media used are primarily those offered by Winogradsky and Omelianski in their first work on isolation, combined with certain modifications. A few new methods will be outlined, some possibly not practical for other lines of work but primarily adapted to the isolation of the nitrifying organisms.

Liquid media. All solutions were prepared from conductivity water and Merck's highest-purity chemicals. The following solutions were used throughout the work:

For the cultivation of the nitrite-forming organism:

	grams
(a) Ammonium sulfate ($(NH_4)_2SO_4$)	1.0
Di-potassium phosphate (K_2HPO_4)	1.0
Sodium chloride (NaCl)	2.0
Magnesium sulfate ($MgSO_4$)	0.5
Ferric sulfate ($Fe_2(SO_4)_3$)	Trace
Water (conductivity)	1000.0
Magnesium carbonate ($MgCO_3$)	Excess

grams

(b) Magnesium ammonium phosphate (MgNH$_4$PO$_4$.6H$_2$O)................. 2.0
   Di-potassium phosphate (K$_2$HPO$_4$)................................ 1.0
   Sodium chloride (NaCl).. 2.0
   Magnesium sulfate (MgSO$_4$).. 0.5
   Ferric sulfate (Fe$_2$(SO$_4$)$_3$)................................. Trace
   Water (conductivity)... 1000.0
   Magnesium carbonate (MgCO$_3$)..................................... Excess

(c) Sodium ammonium phosphate (NaNH$_4$HPO$_4$.4H$_2$O).................. 3.4
   Potassium chloride (KCl)... 2.0
   Magnesium sulfate (MgSO$_4$)....................................... 0.5
   Ferric sulfate (Fe$_2$(SO$_4$)$_3$)................................ Trace
   Water (conductivity).. 1000.0
   Magnesium carbonate (MgCO$_3$).................................... Excess

For the cultivation of the nitrate-forming organism:

(d) Sodium nitrite (NaNO$_2$)... 1.0
   Sodium carbonate (Na$_2$CO$_3$).................................... 1.0
   Di-potassium phosphate (K$_2$HPO$_4$)............................. 0.5
   Sodium chloride (NaCl).. 0.5
   Magnesium sulfate (MgSO$_4$)...................................... 0.3
   Ferric sulfate (Fe$_2$(SO$_4$)$_3$).............................. Trace
   Water (conductivity)... 1000.0

Solution (a) was used for the cultivation of *Nitrosomonas* throughout most of the work and (b) and (c) used in comparison. Unless otherwise specified it will be understood that solution (a) was used.

All glassware was cleaned by treating it with strong alkali, strong sulfuric acid cleaning mixture, then washing in water, and finally rinsing in the conductivity water. The solutions were prepared in liter Erlenmeyer flasks, then pipetted into 150-cc. flasks and sterilized at 15 pounds pressure for 15 minutes. In solutions (a), (b) and (c), all the ingredients were added before sterilization except the magnesium carbonate, which was sterilized in a separate vessel and added to the solutions after they had cooled. During the first part of the work 25-cc. portions of the solutions were placed in the flasks; later on 15-cc. portions were used, because this afforded better aeration and therefore more rapid nitrification. In some cases the magnesium carbonate was added along with the other salts before sterilization and the ammonium sulfate sterilized in separate solution and added to the flasks by means of a sterile pipette.

Gypsum blocks. Gypsum blocks were prepared by mixing 2 gm. magnesium carbonate with 100 gm. of calcium sulphate, (CaSO$_4$.H$_2$O), and sufficient water to make a putty-like mass. This mixture was spread over a smooth glass surface to the desired thickness, marked out in squares, and allowed to dry. When dry the blocks were placed in petri dishes, sterilized, and the desired liquid medium added.

Washed agar. The agar was prepared by washing Difco agar in distilled water for several days and drying at 60°C. From the washed agar a 2.5 per cent solution was made, tubed in 10 cc. portions and sterilized in the autoclave

at 15 pounds pressure.   The following solutions were prepared and sterilized in small portions, about 100 cc. per flask.

	gm. per 100 cc.
(1) Di-potassium phosphate (K_2HPO_4)	1.5
(2) Sodium nitrite ($NaNO_2$)	1.5
Sodium carbonate (Na_2CO_3)	1.5
(3) Magnesium sulfate ($MgSO_4$)	0.45
Sodium chloride ($NaCl$)	0.75
Ferric sulfate ($Fe_2(SO_4)_3$)	0.02
(4) Ammonium sulfate (($NH_4)_2SO_4$)	1.5
Magnesium sulfate ($MgSO_4$)	0.75
Ferric sulfate ($Fe_2(SO_4)_3$)	0.02
(5) Sodium chloride ($NaCl$)	3.0
Sodium carbonate (Na_2CO_3)	1.5

The agar was melted and cooled at 40°C. *Nitrobacter* plates were made by placing 1 cc. each of solutions (1), (2) and (3), in sterile petri-dishes, adding the desired inoculum, and then the melted agar.   For the cultivation of *Nitrosomonas* 1 cc. each of solutions (1), (4) and (5) plus the agar were used.   These stock solutions are about 15 times the strength of the usual medium; so that when 1 cc. of each of three are mixed with the 10 cc. of agar, all the nutrient salts are present in the proper proportions.   The concentration is determined by the amount of agar added which may be controlled at will. The solutions prepared in this manner are convenient for use in agar or silicic acid gel.   The nutrient salts may be added to the agar before autoclaving, but in this case the ammonium sulfate must be sterilized separately, otherwise a part of the ammonia will be lost in sterilization.   The sodium carbonate should be handled in similar manner to prevent hydrolysis of the agar.   Since these two ingredients must be handled separately it is well to prepare the solutions as stated above.   In order to prevent the precipitation of the salts, it is necessary to handle the solutions as outlined.   When preparing the plates it is desirable to prevent the 1-cc. portions of the different solutions from mixing before the agar is added to the plate; if this is attained the plate will contain no precipitated material and the slightest growth can be detected. If it is desirable to use magnesium carbonate as the base for the cultivation of *Nitrosomonas*, the sodium carbonate may be omitted from solution (5) and magnesium carbonate added to the plate at the time of pouring.   Both magnesium and sodium carbonates were used in this work, but the latter gave better results because the plate remained clear and the colonies could be detected more readily.

No egg albumen was added to the agar to aid in clarification.

The agar was heated in the steamer for several hours, and filtered through macerated filter paper under pressure until it was clear.

Silicic acid gel. A convenient method for the preparation of silicic acid gel for use as a culture medium has never been devised. During the first part of the work the method of Stephens and Temple (54) was employed. The preparation of the medium by this method was very pleasing because of its ease, but the results secured from its use were very unsatisfactory. In no case did the nitrifying organism produce growth on this medium.

Doryland's (16) method was also tested but poor results were obtained. This was perhaps due to the high concentration of salts which resulted in an osmotic pressure that could not be withstood by the organisms. The Doryland method is very promising and should bring good results when worked out more in detail.

The method of Winogradsky also was employed, with collodion sacks as a dialyzing membrane, but gave results too irregular to be practical. Silicic acid secured by the Winogradsky method is difficult to handle because of its concentration; it often forms the gel in the dialyzing process, or gels when sterilized. It is interesting to note that the method of Stahel (53) is the same as that of Winogradsky except that the former used a sodium silicate solution with a specific gravity of 1.10, a 10 per cent solution, while the latter used a solution of 1.05 specific gravity, a 5 per cent solution. They each used a parchment membrane but the permeability must have been different because the resultant acid secured in each case was the same, or practically the same. The results bring out the fact that the membrane is the most important single factor, which is emphasized by Winogradsky.

The method of Beijerinck (7) was used with fairly satisfactory results. It is as follows: Merck's 40 per cent sodium silicate solution is diluted to 8 per cent, and a solution of hydrochloric acid prepared of such a strength that 1 cc. is just neutralized by 1 cc. of the 8 per cent sodium silicate solution, with methyl orange as indicator. A 5-cc. portion of the hydrochloric acid is then drawn from a burette into a sterile petri dish, and the amount of sodium silicate required to neutralize the acid added from a second burette. If the two solutions have been allowed to stand in the burettes for some time, they will be practically sterile. This method gives a beautiful gel in about 2 or 3 minutes. The silicic acid gel is then dialyzed by pouring sterile water over the surface until all the chloride has been removed. The entire procedure requires but a few hours for a large number of plates. The sterile concentrated nutrient solution is now poured on the surface of the plate and allowed to diffuse through the gel for some time; the excess is poured off and the surface of the gel flamed to insure that it is sterile. The plates are inoculated by placing a drop of the inoculum on the surface of the medium and spreading it around with the blunt end of a glass rod. The greatest objection to this method is the fact that the plates must be inoculated by streaking the surface, which is very difficult to do without disturbing the smooth surface of the gel, and the character of growth secured is not satisfactory for the study of single colonies.

It was desirable to have a gel which could be inoculated before solidifying, therefore dilute solutions were resorted to. . The method used was primarily that of Schramm (52). Ninety cubic centimeters of Merck's 40 per cent sodium silicate solution are diluted to 1 liter with distilled water, and 125 cc. of concentrated HCl are diluted to 1 liter, and the two solutions poured together. It is necessary to pour the sodium silicate solution slowly into the hydrochloric acid solution, with vigorous stirring, or the silicic acid will precipitate immediately. The mixture, which should have no turbidity or cloudiness, is then placed in a dialyzer fitted with a parchment paper in contact with running water. After several days, 8 to 10, the mixture should be entirely free from chlorides, as determined by the silver nitrate test. It is then dialyzed further with several changes of distilled water, and a 10-cc. portion removed, evaporated to dryness, and ignited, and weighed to determine the percentage of silicon dioxide (SiO_2). If it is desirable to determine the purity of the acid, it may be done by moistening the residue with sulfuric acid, volatilizing the silica with hydrofluoric acid (HF), and reweighing. The dialyzed mixture usually gives an acid containing 1 to 1.5 per cent of silicon dioxide (SiO_2), Variations depending on the quality of the membrane used will occur. Since this solution of colloidal silicic acid is too dilute to produce the gel it must be concentrated, but if concentrated it will solidify on sterilization. This difficulty is met by sterilizing before concentrating. The acid solution is placed in a suction filter flask which is fitted with a thermometer and a capillary tube, which in turn is connected with a carbon filter plugged with cotton and is controlled with a pinchcock. The flask so arranged and containing the solution is then sterilized by the intermittent method. It is then attached to a suction pump and the pressure reduced to such an extent that the solution boils at 40 to 50°C. The capillary tube is opened slightly to permit a very slow stream of air to pass through the solution, but not sufficiently rapid to raise the pressure within the flask. The nutrient salts to be used with the silicic acid will determine how concentrated it must be to get the proper gel in the desired length of time. In this case the solutions given on page 26 were used, as previously described in connection with agar, and required an acid of 1.5 to 2.5 per cent of silicon dioxide (SiO_2). The addition of the salts causes the formation of the gel. Tests may be made as the silicic acid is concentrating by removing 10-cc. portions from the flask and adding the desired salts. When sufficiently concentrated to produce a gel of the desired consistency in a few minutes' time, the process is discontinued. In some cases it will be possible to sterilize this silicic acid solution after concentrating, either in the autoclave or by the intermittent method, without producing the gel. If this can be done the entire lot should be sterilized in suitable containers. If too concentrated to withstand sterilizing further, it must be used direct from the concentrating chamber. A clear solution is absolutely essential. If it takes on an opalescence or cloudiness it is always unsatisfactory because it will either not stand sterilization or will not form the gel when the desired salts are added.

It is a well known fact that the addition of electrolytes to a solution in the colloidal state tends to precipitate or bring about the gel condition. Since the nutrient solutions used in this work were all made up of electrolytes, no difficulty was experienced in obtaining the gel. If the medium is to contain organic nitrogenous matter, a higher concentration of the silicic acid must be used because of the protective action of the protein material. Stronger concentrations must also be used with a medium containing sugar because of the fact that carbohydrates in general produce an effect opposite in action to that of the electrolytes. "Reciprocal coagulation" also offers a means of obtaining a gel using more dilute silicic acid, but at present no work has been done in this direction.

It is important to note one interesting point in the preparation of silicic acid. Apparently under the same conditions the same silicic acid is not produced. On several occasions two portions of acid have been secured which contained the same percentage of silicon dioxide (SiO_2); one of these acids gave a gel when concentrated to 2 per cent of silicon dioxide (SiO_2) and would not stand sterilizing at that concentration, while the other was concentrated to 2.6 per cent and withstood heating in the autoclave several times at that concentration, and remained in the sol condition for several weeks. It gave a very beautiful gel of the desired consistency when mixed with the nutrient solutions. The results obtained bear out the fact that acids in various states of hydration may be secured, which act differently towards conditions commonly employed in media making. This would indicate a wider application of the method of preparation outlined by Doryland than has at present been worked out.

Each person using this medium must work out the details of the method of preparation on the general principles which have been laid down. The method will depend upon three factors—the silicic acid present, the concentration and the nutrient medium used. During the course of this work silicic acid gel plates containing the following constituents have been prepared along with the other ingredients.

Heyden Nährstoff
Mannite
Asparagin and dextrose
Dextrose and nitrate

Soil counts were made on these media with good results. *Azotobacter* developed readily on the mannite gel and produced the usual pigment. Large tubes of soft silicic acid gel also were prepared for the cultivation of plants and studying nodule formation, but the gel was not satisfactory for root development. Apparently the plant roots cannot penetrate this silicic acid gel medium.

RESULTS OF INVESTIGATION

In the following discussion the results of the work with *Nitrobacter* are presented first. The experience gained in the isolation of *Nitrobacter* aided materially in the isolation of the nitrite-forming organism.

The liquid cultures secured by inoculation with soil and carried in the enrichment process are referred to as "crude cultures," corresponding to the term "Rohkulturen" used by Winogradsky and other investigators.

Crude cultures of nitrobacter

Enrichment process. Flasks containing 25 cc. of the nutrient solution (d, p. 437) were each inoculated respectively with 0.5 gm. of garden, neutral and acid soil from widely separated sources. These flasks were incubated at 28°C., and after four or five days were tested with Trommsdorf's reagent until the absence of nitrites was noted. These cultures were then tested with di-phenylamine and sulfuric acid for the presence of nitrates. Wherever the nitrite was converted to nitrate, two or three loops were transferred to fresh flasks of the same medium. The mother culture was then covered with lead-zinc foil to prevent evaporation and held at room temperature, while the subcultures were incubated at 28°C. This procedure was continued throughout the work. The results are shown in table 1.

From the data in this table it is apparent that the time required for the complete oxidation of the nitrite varies with the different generations. Since the solutions were prepared in the same manner each time, and many of the generations are from one batch of medium, and the conditions of incubation are the same, it is safe to conclude that the variation in time for oxidation is due largely to the variation in the number of organisms carried by the loop inoculation. This difference in number will be further brought out in a later table. The cultures do not show any inclination to lose their virulence as a result of the enrichment process; they are still on hand and show their usual activity. The source of the culture has little influence on the time of oxidation, at least the three soils chosen for this test required practically the same time for oxidation.

The data in the table indicate that there are several cultures which required an exceptionally long period of time for oxidation. The long period of time required for oxidation of the neutral soil cultures F_{12} and F_{21} cannot be explained. Apparently the trouble in the F_{21} culture was not in that particular culture but in the F_{20} culture, because several F_{21} cultures were made before one was secured which would produce oxidation and this only after the lapse of 52 days.

Number of bacteria in enrichment cultures. Dilution counts were made to determine the number of organisms in soil capable of oxidizing nitrite in solution, and likewise the number present in the enrichment cultures. The

TABLE 1

Time required for oxidation of the sodium nitrite by the successive generations of nitrobacter

NUMBER OF GENERATION	TIME FOR OXIDATION		
	Neutral soil	Garden soil	Acid soil
	days	days	days
F	22	23	15
F_1	18	20	Date not recorded
F_2	9	6	9
F_3	10	9	10
F_4	8	12	7
F_5	9	11	12
F_6	10	9	12
F_7	11	8	10
F_8	11	8	7
F_9	14	9	7
F_10	16	10	10
F_11	13	10	8
F_12	35	10	14
F_13	12	10	19
F_14	10	11	30
F_15	17	15	15
F_16	14	12	15
F_17	16	30	14
F_18	23	12	17
F_19	19	20	34
F_20	21	10	15
F_21	52	15	13
F_22	14	9	21
F_23	12	12	22
F_24	11	8	21
F_25	10	27	8
F_26	8	16	5
F_27	13	9	27
F_28	9	11	7
F_29	11	7	7
F_30	10	6	8
F_31	10	8	7
F_32	10	6	7
F_33	9	6	7
F_34	7	7	10
F_35	7	7	7

results secured from the soil dilutions were unusually low. Flasks of the usual nutrient solution failed to produce oxidation when inoculated with 1 cc. of a 1:100 dilution from either the acid or neutral soil. The 1:10 dilution in each case produced oxidation after an incubation of 21 days. The results with the dilutions from enrichment cultures are shown in table 2.

The dilutions were made in sterile distilled water and 1 cc. of each dilution was used as an inoculum. From these dilution tests three facts are estab-

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lished; first, soil contains relatively few active organisms capable of bringing about the oxidation of sodium nitrite in solution; second, the enrichment process increases the number of nitrite-oxidizing organisms to more than 1,000,000 per cubic centimeter; third, the time for oxidation depends upon the number of organisms introduced in the inoculum. The rapidity of oxidation in the dilutions from F_{10} would indicate that 1,000,000 bacteria per cubic

TABLE 2

Number of nitrobacter organisms in the enrichment cultures

SOURCE	DILUTION 1 : x	DAYS FOR OXIDATION
	cc.	
	1 cc. undiluted	2
	10	3
	100	6
F_4 Acid soil..............................	1,000	10
	10,000	12
	100,000	14
	1,000,000	No oxidation
	10,000,000	No oxidation
	100	8
	1,000	8
	10,000	14
	100,000	13
F_9 Neutral soil............................	200,000	13
	400,000	13
	600,000	15
	800,000	15
	1,000,000	18
	100	8
	1,000	13
	10,000	15
	100,000	18
F_{10} Neutral soil...........................	200,000	19
	400,000	19
	600,000	20
	800,000	20
	1,000,000	20

centimeter was a very low figure in comparison to the number actually present. However, higher dilutions than this were not made.

Heyden agar plates were inoculated from these same dilutions in order to determine the number of foreign organisms in the enrichment cultures. Colonies developed on the plates from the highest dilutions. The colonies from the higher dilutions were apparently of only two or three types, one a small, round or oval colony, yellow in color, the other a milky colony, or light gray, having a dull appearance, and many times as large as the small yellow colony.

The small type of colony was much more numerous on all the plates. On the plates from the higher dilutions of the F_{10} enrichment only a few colonies developed, and these were apparently of the two types named. The plates from the lower dilutions produced abundant growth and showed the presence of putrefactive organisms.

As a result of these dilution experiments it is safe to conclude that not only does the enrichment process increase the number of nitrifying organisms but that it eliminates a large number of the other types. However, the enrichment process coupled with dilution will not give a pure culture of this organism.

The effect of silicic acid sol on enrichment cultures

A series of flasks of the usual sodium nitrite liquid medium were inoculated from an active enrichment culture. Pure colloidal silicic acid, 1.5 per cent of silicon dioxide, was then added in amounts of 2, 4, 6, 8, and 10 cc. per flask containing. 15 cc. of liquid medium giving 0.176 per cent, 0.310 per cent, 0.428 per cent, 0.522 per cent and 0.60 per cent of silicon dioxide, respectively. These were then incubated at 28°C. Oxidation took place promptly in each, showing the silicic acid did not affect the activity of the organisms. This test was made because of the fact that a great many of the silicic acid plates inoculated gave slow growth and irregular results.

Effect of soil extract on enrichment cultures. Soil extracts from each of the three soils, neutral silt loam, acid silt loam, and garden soil high in organic matter, were prepared by heating 1 kgm. of soil with 1 liter of water in the steamer, then filtering and diluting the filtrate to 1 liter. These extracts were used for the preparation of the sodium nitrite nutrient solution for the cultivation of *Nitrobacter* and the flasks inoculated with an active enrichment culture. Oxidation resulted in each of the flasks with the usual promptness, showing the extracts not to be toxic. This is in accord with the results of Allen and Bonazzi (2) who also found that soil extract was not injurious to the enrichment cultures of *Nitrobacter*.

Isolation of Nitrobacter

Agar plates. Plates of washed agar were prepared as previously described, inoculated from one of the enrichment cultures, then placed in a moist chamber and incubated at 28°C. A period of 10 days to 2 weeks was necessary for the development of *Nitrobacter* on the plates. At the end of this time tests with Trommsdorf's reagent and diphenylamine, showed that the nitrite had been oxidized to nitrate. The growth was scarcely visible to the naked eye but when examined under the microscope numerous small light brown colonies could be seen. Some were round, others oval, and others three-cornered, depending upon their position in the agar. All the colonies were very small and almost any colony on the plate would answer the description of the

Nitrobacter colony given by one or more investigators. The deep colonies were regular in outline but the surface colonies were more or less irregular. A fresh portion of sodium nitrite was added to the plates by removing small portions of the agar and adding the sterile solution to the wells formed in the agar. The addition of nitrite caused the colonies to increase in size but no one type of colony developed faster than the other. At first it was assumed that all the colonies were *Nitrobacter*. This assumption, however, was not true as shown by transfers into liquid media.

Method of securing a single colony. The colonies were too small to make it practical to attempt the isolation of a single colony by means of the platinum needle. This difficulty was met by using a modification of the Barber (57) method of isolation. A small moist.chamber, open at one end and the top, was made by sealing glass slides together with balsam. A capillary tube was then prepared as described by Barber, except that in this case it was not necessary to secure such a fine point. A long sterile cover glass was then slipped under the agar on the plate and a strip removed. This was allowed to remain on the glass and was trimmed to proper size, then the cover glass inverted over the moist chamber in such a manner that the agar was suspended on the slide, as in the hanging drop. The capillary tube was fitted into the mechanical holder and the point brought into focus under the lens, the 16-mm. objective being used. The capillary pipette was lowered and the moist-chamber brought into place under the objective. The chamber was moved about until a desirable colony was located and brought into the center of the field, then the pipette brought upward by the mechanical holder until the point came in contact with the colony. The colony readily moved into the pipette by capillarity—often the opening in the pipette was as large as the colony itself. The colony thus secured was transferred to the usual nutrient solution and growth measured by oxidation.

This process seems simple and yet many attempts failed before the organism was secured in pure culture. Usually the organism secured from the agar in this manner failed to oxidize the nitrite in the solution, or if oxidation took place subsequent inoculation into bouillon resulted in growth. Many colonies in isolated positions on the medium were secured in this manner and when inoculated into the usual nutrient solution produced oxidation, yet the bouillon test after oxidation showed contamination. Either the contaminating organism was very closely associated with the nitrate-forming organism and the colony characteristics were so nearly alike they could not be differentiated, or the nitrate-forming organism produced growth in bouillon as claimed by Beijerinck (7). A large percentage of the colonies transferred to the nutrient solution resulted in no oxidation of the nitrite, yet a very small piece of the agar 5 mm. square readily produced oxidation when placed in the same solution. This would indicate that a large inoculum was necessary as is the case with certain other organisms. Two colonies which appeared identical were chosen, one transferred to a nutrient solution, the other to bouillon. Neither

gave evidence of activity, the nitrite remaining unchanged in the solution, the bouillon remaining clear. This would indicate a third organism, which would neither grow in bouillon or oxidize the nitrite in the nutrient solution, as claimed by Stutzer and Hartleb. However, this is not considered to be true in this case, but is explained by the fact that growth in bouillon results only from use of a large inoculum. The small percentage giving oxidation of the nitrite may be explained in a similar manner. This will be further discussed with the results of the *Nitrosomonas* study.

To narrate all the difficulties which arise in the isolation of *Nitrobacter* would be a difficult task and without interest to the reader. The following translation from the report of Stutzer and Hartleb (57) quite accurately expresses the sentiment of the author in regard to the difficulties of isolation, more so in connection with the isolation of *Nitrosomonas* than with *Nitrobacter*. However, this quotation deals with the isolation of *Nitrobacter*.

Simple as the described method for the isolation of the nitrate former may appear, which varies little in its technique from the customary bacteriological methods of securing a pure culture, and differs from these mainly only in the application of other nutrient media, yet the practical application of it is very round-about and requires much time, because very often difficulties arise which make the undertaken work absolutely useless, or if one believes that he is at his goal a new difficulty suddenly appears. For instance, such a difficulty was found in the separation of the two organisms which would not grow in bouillon, *Hyphomicrobium* and *Nitrimicrobium*. As long as we did not know that several bouillon sterile organisms occurred in the soil we were inclined to consider the two kinds mentioned as identical, inasmuch as also their colony formation and their action on nitrite agar show a surprising similarity. . . . . And we may comfort ourselves with the thought that up to this time no other investigator has been successful by arriving completely at his goal in this special field.

Silicic acid gel plates. The results secured from the use of silicic acid gel were more satisfactory in the earlier part of the work of isolation than those secured from the use of washed agar. However, the same disappointment may be felt when the isolation is begun. The silicic acid gel is not well adapted to the cultivation of organisms other than those requiring an inorganic medium, which makes it a very valuable solid medium for the cultivation of the nitrifying organisms. After an incubation of 10 days to 2 weeks the nitrite in the medium was all oxidized. When examined under the microscope numerous very small light brown colonies were seen, some of which were not *Nitrobacter*, but after the selection of a great number as described above, a pure culture was obtained. The colonies remained much smaller than those on the agar, although more nitrite was added repeatedly.

Tests for purity. The fact that colonies from agar or silicic acid gel, secured as above outlined, resulted in oxidation of the nitrite in solution, was not taken as an absolute proof of the purity of the culture. Further tests were made by inoculation into bouillon and upon agar slopes. The bouillon tubes were incubated 8 to 10 days because the growth of the contaminating forms devel-

oped very slowly.   Agar containing 3 gm. of beef extract and 5 gm. of peptone per liter gave better results than bouillon.   The growth on the agar was visible more quickly than in bouillon.   The type of growth is very slight in comparison to the growth secured with common soil organisms.   When bouillon was used as the test for purity, 0.5 cc. of the culture was used as inoculum.   It was found that loop inoculations often fail to show the presence of the contaminating organisms.   The contamination usually consisted of two organisms which also oceurred with *Nitrosomonas* and will be described in connection with the discussion of that organism.

Pure culture characteristics of Nitrobacter

Liquid cultures.   When the nutrient solution used in the *Nitrobacter* enrichment process was inoculated with a pure culture of the organism, complete oxidation of the nitrite resulted in about 7 days.   At the end of that time, the solution contained no visible growth.   After the addition of several portions of sterile 1.5 per cent sodium nitrite solution, with subsequent oxidation of each, a very slight flocculent material began to accumulate.   This material always settled to the bottom of the solution and in no case formed a film on the surface or developed on the sides of the flask.   When removed and stained with carbol fuchsin it was found to consist of masses of the *Nitrobacter* cells together with disintegrated material.   The amount of this material was small.   The oxidation of the nitrite solution continued with the addition of each portion until the concentration of nitrate became surprisingly high.

The organisms were readily stained with carbol fuchsin.   Loeffler's methylene blue and aqueous gentian-violet were tried without success.   The organism is distinctly oval in shape, commonly found in single cells or in pairs, about 0.6 to 0.8 μ wide and 1 to 1.2 μ in length.   It is non-motil and non-spore-forming.   The thermal death-point is between 56° and 58°C.   Stains of the typical forms are shown in plates 2 and 3.

Colonies on washed agar.   Agar plates inoculated with pure cultures of the organism produced colonies which were first identified under the microscope after an incubation of 7 to 10 days at 28°C.   They appeared as very small light brown colonies more or less rounded in outline.   After the nitrite was completely oxidized to nitrate, usually 10 days to 2 weeks or even longer, the colonies developed somewhat larger.   They then appeared darker in color, round or three-cornered in shape, and with a regular outline.   The surface colony was occasionally regular in outline but was more often irregular and spreading.   The deep colony at this stage was 30 to 50 μ in diameter; the surface colony somewhat larger, 50 to 150 μ in diameter, and with a tendency to spread.   The deep colony appeared homogeneous and somewhat shiny, the surface colony slightly granular and dull.   The typical deep colony on agar is shown in plate 1.

Colonies on silicic acid gel plates.   The colonies on the silicic acid gel were very similar to those on washed agar, being somewhat more dense and not as large.   They had the characteristic light brown color, later becoming dark brown.   When silicic acid gel plates were prepared after the method of Beijerinck and inoculated by streaking the surface, growth was more profuse and resembled that on the agar slope.   To the naked eye it appeared as very scanty gray streaks over the plate.   When examined under the microscope the streak was found to be made up of a mass of growth, light brown in color, around the edge of which could be seen many very small light brown colonies. When small portions of the gel were removed and sterile sodium nitrite added to the wells thus formed, the growth increased and became a characteristic dark brown color when examined under the microscope.   To the naked eye the growth remained a light gray color throughout the period of activity of the organisms.

Washed agar slopes.   When a slope of washed agar containing the nutrient salts was inoculated with a pure culture in liquid medium, growth was visible after a period of 4 to 5 days, and after a period of 7 to 10 days formed a scanty greyish streak on the surface.   Growth was more uniform after a few transfers, the first growth secured being somewhat scattered.   When the slopes were inoculated from cultures in liquid media the resultant growth was often in the form of numerous very small colonies on the surface of the agar.   Continuous growth was secured from this by streaking the surface of the slope with the platinum needle, thus joining the colonies.   The usual nutrient solution when inoculated from the slope was promptly oxidized.

Accumulation of nitrate in liquid cultures.   Two 1-liter Erlenmeyer flasks each containing 100 cc. of the usual sodium nitrite nutrient solution were inoculated with a pure culture of *Nitrobacter* and incubated at 28°C.   When tests with Trommsdorf's reagent showed no nitrite to be present 1 cc. of sterile 10 per cent sodium nitrite was added to each flask.   After the oxidation of the first portion of nitrite the solutions remained quite clear, but after the addition of one or two portions of the 10 per cent solution the flaky growth began to appear.   Stains then showed the presence of an enormous number of the *Nitrobacter* cells.   The flaky material developed for some time, then remained at about the same stage without further development.   Each time the nitrite was oxidized more was added and the process continued until no further oxidation could be secured.   Analysis of the solutions at the end of the period showed them to contain 502 and 527 mgm. of nitrogen, respectively, as nitrate per 100 cc. of solution.   Stains at a late stage of oxidation showed the presence of fewer organisms than after the addition of the third portion of the nitrite solution.   After all oxidation ceased transfers were made to fresh media and oxidation resulted promptly, showing that the organisms were not killed by the products of their own metabolism but a concentration was reached beyond which no further activity took place.

Thermal death-point. The thermal death-point was determined by use of actively oxidizing pure cultures in liquid media. Tubes containing 10 cc. of a 0.7 per cent sodium chloride solution were placed in the water baths at various temperatures and held until of uniform temperature throughout. Then 0.5 cc. of the culture was pipetted into each tube, the contents thoroughly shaken, and held at the temperature for 10 minutes, and then 1 cc. was drawn from the tube and used as inoculum in the usual sodium-nitrite-nutrient solution. All tests were run in duplicate at different times, different cultures being used. The thermal death-point was found to be between 56° and 58°C.

Longevity. Two samples of soil were available which had been on hand in the laboratory since October 28, 1910. This soil had been taken from the field on that date, air-dried, passed through a 100-mesh sieve, and then stored in tightly stoppered bottles. This soil was used as inoculum in the usual nutrient solution February 3, 1917. Oxidation of the nitrite resulted promptly in each flask inoculated, showing the resistance of the organisms to drying.

Ten grams of soil were placed in each of a number of test tubes and sterilized in the autoclave at 10 pounds pressure for 1 hour on each of three consecutive days. One cubic centimeter of sterile sodium nitrite was then added to each tube. The tubes were then divided into two series; one series was brought to optimum moisture content, approximately, and the other series was unchanged. The tubes of the series with the optimum moisture content were then sealed to prevent loss of moisture while those of the first series were left with cotton plugs. Both series of tubes were then held at room temperature to determine how long the organisms would retain their vitality, as determined by their activity when inoculated into the usual nutrient solution.

The organisms remained active in all the tubes, both sealed and plugged with cotton, for a period of 4 to 5 months. They then began to show less activity in that it required a longer period of time for the oxidation of the nitrite when inoculated into the nutrient solution. The last test was made 17 months after the tubes were stored and at that time the organisms were present and still able to oxidize. Bouillon tubes inoculated from the soil remained free from all growth.

Crude cultures, Nitrosomonas

Enrichment cultures. The flasks containing the ammonium sulfate nutrient solution (a, p. 436), were each inoculated with 0.5 gm. of the three soils previously described. They were then incubated at 28°C., and tested every second or third day with Trommsdorf's and Nessler's reagents. When the test with Trommsdorf's reagent gave a deep blue color loop transfers were made to fresh media, and the process continued. The results of the enrichment process are shown in table 3.

From the data in the table it is apparent that the time required for the oxidation of the ammonia in the different generations is variable. In general the

TABLE 3

Time required for the oxidation of ammonium sulfate by the successive generations of nitrosomonas

GENERATION NUMBER	TIME FOR OXIDATION		
	Neutral soil	Garden soil	Acid soil*
	days	days	days
F	7	7	7
F_1	8	21	8
F_2	5	9	5
F_3	8	10	15
F_4	6	53	4
F_5	6	61	6
F_6	6	6	6
F_7	6	8	6
F_8	6	7	6
F_9	7	7	6
F_{10}	8	16	7
F_{11}	9	12	8
F_{12}	8	20	9
F_{13}	8	15	8
F_{14}	6	10	8
F_{15}	13	9	6
F_{16}	10	11	13
F_{17}	20	40	10
F_{18}	9	6	11
F_{19}	6	7	10
F_{20}	9	7	6
F_{21}	10	4*	6
F_{22}	9	6	9
F_{23}	5	6	9
F_{24}	16	7	9
F_{25}	16	6	5
F_{26}	10	4	19
F_{27}	8	7	31†
F_{28}	7	7	9
F_{29}	9	7	7
F_{30}	7	7	8
F_{31}	7	7	8
F_{32}	7	8	6
F_{33}	6	8	7
F_{34}	4	8	7
F_{35}	7	7	7
F_{36}	8	7	7
F_{37}	7	10	7
F_{38}	7	7	6
F_{39}	7	8	4
F_{40}	8		7
F_{41}	8		7

* Garden soil series. 15 cc. medium substituted for 25 cc. and maintained in rest of cultures.

† First F_{27} failed to oxidize the ammonia, second required 31 days.

TABLE 3—*Continued*

GENERATION NUMBER	TIME FOR OXIDATION		
	Neutral soil	Garden soil	Acid soil
	days	*days*	*days*
F_{42}	7		7
F_{43}	7		7
F_{44}	7		7
F_{45}	10		8
F_{46}	6		8
F_{47}			6
F_{48}			7
F_{49}			6
F_{50}			10
F_{51}			6
F_{52}			7

time becomes more uniform in the higher generations, as was found with the *Nitrobacter* cultures. The fluctuation in most cases is probably due to the variation in the number of nitrite-forming organisms transferred. No explanation can be given for the extreme length of time required for the oxidation of the ammonium salt in a few of the cultures, since all media were prepared in the same manner and all cultures were handled alike. The garden soil series was very variable until the F_{21} generation. At this point 15 cc. of the nutrient solution was substituted for 25 cc. previously used. The increased surface, in proportion to the depth of the solution, was responsible for the more vigorous oxidation of the ammonium salt. In the acid soil series the F_{27} generation failed to oxidize the ammonium and it was only after several transfers were made that a culture was secured which was active. The point of interest in this connection is the fact that there was no loss of oxidizing power from transfer to transfer as the cultures were continued. This is not in accord with the results secured by Allen and Bonazzi (2), who found that the cultures became less active in the successive transfers. In their first paper the cultures became so weakened after three or four transfers that they were forced to discontinue the process.

After a few generations of enrichment cultures had been secured, stains with carbol fuchsin were made to determine the relative number of organisms present. In the majority of cases the stained preparations showed relatively few organisms but if the material for staining was taken from the bottom of the flask numerous clumps of organisms could be seen. These were in general about 0.9 to 1 μ wide and 1.2 to 1.5 μ long, rounded or oval, and uniformly stained. Few other types of organisms were present, but if the smears were examined carefully a very small rod form could be found and also an occasional clump of a very small coccus form. Sterile ammonium sulfate solution was then added to the cultures and was promptly oxidized to nitrite in two or three days, and each successive portion added was likewise oxidized. It

was necessary to add more magnesium carbonate from time to time as the concentration of nitrite increased. No growth in the culture solutions was visible until the nitrous acid formed had rendered the magnesium carbonate soluble, and then only a very slight flaky material could be seen. In no case did the organism grow on the surface of the solution or on the sides of the flask. When stains were prepared from the cultures after the oxidation of several added portions of ammonium sulfate, the number of organisms was found to have increased enormously. The stains showed the same type of organism previously observed but in much greater numbers, both in clumps and scattered as free cells over the field. The contaminating organisms were either in such a minority or so nearly resembled the nitrite-forming organism that the stain indicated the cultute was pure. However, this was not the case, as will be explained later. The nitrite content gradually increased, as each successive portion of ammonium sulfate was oxidized, accompanied by a large increase in the number of organisms up to about the seventh or eighth addition, beyond which the cells began to disintegrate, probably because of the high concentration of nitrite. After the addition of eight or nine portions of ammonium sulfate solution the time required for oxidation increased materially and after the addition of 12 to 15 portions the process was entirely inhibited.

Flasks containing the nutrient solutions of magnesium ammonium phosphate (b, p. 437) and sodium ammonium hydrogen phosphate (c, p. 437) were inoculated from one of the enrichment cultures, and after oxidation of the ammonia had occurred transfers were made in the usual manner. These cultures were carried through a number of generations in order to determine whether or not there was any advantage in the use of these salts as the source of ammonia. The results showed that the time required for the oxidatio n of these salts was much the same as for ammonium sulfate, and if there was any advantage it was in favor of the ammonium sulfate solution.

Number of bacteria in enrichment cultures. In order to determine whether or not the enrichment process was actually increasing the number of nitrite-forming organisms dilution counts were made from the cultures. These dilutions were made immediately after the oxidation of the ammonium sulfate, and before the addition of a second portion. The culture was shaken vigorously in order to get the contents well mixed, and 1 cc. transferred by means of a sterile pipette to a 99-cc. water blank. Successive transfers were made in a similar manner, thus securing the dilutions indicated in table 5. In the case of the F_{31}, acid soil, the dilutions were made direct in the nitrifying media, transferring 1 cc. from each flask to the next. One cubic centimeter of each dilution in the water blanks was used as inoculum in the usual nutrient solution, and in this manner the presence or absence of *Nitrosomonas* in that dilution determined. At the same time Heyden agar plates were inoculated from the dilutions and incubated at 28°C. The results of the *Nitrosomonas* counts are shown in table 4.

From the data in the table it is apparent that the number of nitrite-forming organisms in the cultures has increased from enrichment F_4 to enrichment F_{33}. It is also apparent that the time required for the oxidation of the ammonia was somewhat dependent on the number of organisms used as inoculum. The highest count obtained was 19,000,000, which is about the total number of organisms usually found in 1 gm. of soil as determined by the plate method.

Colonies developed on the Heyden Nährstoff agar plates from all the dilutions, showing contaminating organisms to be present in numbers equally

TABLE 4

Number of nitrosomonas organisms in the enrichment cultures

GENERATION NUMBER	DILUTION 1 : x	DAYS FOR OXIDATION
F_4. Acid soil; dilutions in distilled water; 12/28/15	none, 1 cc.	2
	10	3
	100	6
	1,000	10
	10,000	12
	100,000	14
	1,000,000	No oxidation
	10,000,000	No oxidation
F_{11}. Acid soil; dilutions in saline; 2/10/16	100	7
	1,000	8
	10,000	14
	100,000	13
	200,000	13
	400,000	13
	600,000	15
	800,000	15
	1,000,000	18
F_{16}. Neutral soil; dilutions in saline; 2/10/16	100	8
	1,000	8
	10,000	9
	100,000	lost
	200,000	15
	400,000	18
	600,000	15
	800,000	19
	1,000,000	22
F_{33}. Acid soil; dilutions in nitrifying solution; 11/7/16	100	6
	2,500	10
	62,500	11
	125,000	13
	781,000	16
	1,562,600	16
	19,031,050	18
	38,062,500	No oxidation

TABLE 4—*Continued*

GENERATION NUMBER	DILUTION 1 : x	DAYS FOR OXIDATION
	100	5
	1,000	7
	10,000	8
	100,000	10
	200,000	10
	500,000	11
F_{11}. Acid soil; dilutions in saline; 11/17/16	800,000	12
	1,000,000	14
	2,000,000	15
	5,000,000	17
	8,000,000	17
	10,000,000	19
	20,000,000	No oxidation
	50,000,000	No oxidation

as great as or greater than *Nitrosomonas*.   The plates from the F_4 generation showed a higher number of colonies than those from F_{10}, indicating an elimination of undesirable types due to the enrichment process.   The plates from the lower dilutions showed five or six types of colonies, those from the higher dilutions developed 4 to 20 colonies each of apparently only two types, one a very small, round or oval colony, yellowish brown in color, the other a light greyish colony of dull appearance, very round in shape, and many times as large as the small yellow colony.   The small colony was far more numerous than the larger type, and sometimes developed a bright yellow color when seen under the microscope.   From the results of these plates it seems that only two organisms are present in the enrichment cultures in as great numbers as *Nitrosomonas*.   These two organisms will be discussed later.

The results of these dilution counts are much the same as those secured with the *Nitrobacter* cultures and the same conclusions may be drawn from their study.   Not only does the enrichment process increase the number of desirable organisms but it eliminates a high percentage of the undesirable types.

Effect of sodium chloride on enrichment cultures.   The poor results secured by the use of silicic acid gel prepared after the method of Stephens and Temple (54) were attributed to too high a concentration of sodium chloride.   Flasks containing the usual ammonium sulfate nutrient solution and sodium chloride at various concentrations were each inoculated from an active enrichment culture, in order to determine the amount of sodium chloride which would exert toxic action on nitrite formation.   The results are shown in table 5.

The solution used for the cultivation of *Nitrosomonas* throughout the work contained 0.2 per cent sodium chloride.   From the data in the table it is seen that concentrations above 0.8 per cent greatly inhibited nitrite formation, and concentrations above 1 per cent prohibited oxidation.   The data indicate that silicic acid gel prepared after the method of either Stephens and Temple

TABLE 5

Effect of sodium chloride on nitrosomonas enrichment cultures

GENERATION	SODIUM CHLORIDE PERCENTAGE	DAYS FOR OXIDATION
	0.2	5
	0.2	5
F_9. Acid soil; 2/8/16......................	0.5	9
	0.5	10
	1.0	No oxidation
	1.0	No oxidation
	0.2	5
	0.2	5
F_9. Neutral soil; 2/8/16..................	0.5	12 to 15
	0.5	12 to 15
	1.0	No oxidation
	1.0	No oxidation
	None	17 ⎱ very
	None	17 ⎰ weak
	0.05	9 ⎱ fairly
	0.05	9 ⎰ strong
	0.10	9 strong
	0.10	9 strong
	0.20	9 strong
	0.20	9 strong
	0.40	9 strong
	0.40	9 strong
F_{16}. 4/14/17............................	0.60	9 strong
	0.60	9 strong
	0.80	12 strong
	0.80	15 strong
	1.00	20 ⎱ very
	1.00	20 ⎰ weak
	1.50	No oxidation
	1.50	No oxidation
	2.00	No oxidation
	2.00	No oxidation

or Doryland is not adaptable to the cultivation of *Nitrosomonas*. The F_{16} culture withstood a higher concentration of the sodium chloride than the F_9 culture, which indicates that continual cultivation in the liquid medium has increased the resistance of the organism to sodium chloride.

Effect of silicic acid on enrichment cultures. Silicic acid, containing 1.5 per cent of silicon dioxide (SiO_2) was added to the flasks containing the nutrient medium, in quantities of 2, 4, 6, 8 and 10 cc., respectively. Thus the flask containing 2 cc. of the silicic acid sol had a total volume of 17 cc. of solution while the flask receiving 10 cc. of the silicic acid had a volume of 25 cc. of solution, giving 0.176 per cent, 0.316 per cent, 0.428 per cent, 0.522 per cent and 0.600 per cent of silicon dioxide, respectively. The flasks were then inoculated from an active enrichment culture. Oxidation resulted promptly in each flask. Varying quantities of pure silicic acid in the gel state were then added to each of another series of flasks which were then inoculated. Oxidation resulted in each flask in the usual time. These results showed that the

TABLE 6

Effect of soil extract on enrichment culture of nitrosomonas

EXTRACT	K_2HPO_4 + or −	OXIDATION
Neutral soil..	+	0
	−	0
Acid soil...	+	+
	−	−
Garden soil..	+	++
	−	++

0 No nitrite formation.
+ Fair nitrite formation.
++ Very strong nitrite formation.

silicic acid either as sol or gel had no toxic action on nitrite formation in solution. Bonazzi found the same thing true with his cultures.

Effect of soil extract on enrichment cultures. Soil extracts from each of the three soils, neutral silt loam, acid silt loam, and garden soil heavy in organic matter, were prepared by heating 1 kgm. of soil with 1 liter of water in the steamer for 1 hour. These were then filtered and washed until the filtrate from each was 1 liter in volume. These extracts were then each divided into two lots, one of which received 1 gm. di-potassium phosphate (K_2HPO_4) per liter, while the other received no treatment. These extracts were then used as the nutrient medium, after the addition of ammonium sulfate and magnesium carbonate in the proper proportion. Both these ingredients were added after the sterilization of the extract. The flasks were then inoculated from an active enrichment culture and tested regularly for the first oxidation of the ammonia. The results are shown in table 6.

The cultures prepared with the garden soil extract gave oxidation as readily as or more readily than those in the usual ammonium sulfate nutrient solution. This was unexpected since the extract was deep brown in color from the soluble organic matter. The acid soil extract was much lighter in color and the neutral soil extract was nearly colorless. The absence of soluble plant-food rather than the toxic effect of the organic matter explains the results of no oxidation in the first two extracts. The garden soil had an abundance of soluble plant-food as well as the large amount of organic matter.

Isolation of Nitrosomonas

Agar plates. The isolation of the organism by use of the plate method was accomplished only by the use of super-enrichment cultures; that is, cultures which had received several additions of ammonium sulfate with subsequent oxidation of each. A drop of sterile water was placed in each of several petri-dishes and a loop of the solution taken from the desired culture and mixed with the water in the first plate, then a loop from this to the second plate, etc., thus securing a greater dilution with each successive plate. The nutrient salts in sterile concentrated solution were then added as previously described, the agar poured into the plate and the contents thoroughly mixed, and then the plate incubated in incubating cylinders at 28°C. The plates were tested at intervals by removing a small piece of the agar and placing it in Trommsdorf's reagent. In case the nitrite formers were active, which in general required an incubation of 2 to 4 weeks, the test would give a deep blue color. When the plates were examined before there was any nitrite formation they were found to contain many colonies, scarcely visible to the naked eye, but visible under the 16-mm. objective. The colonies appeared light brown in color, rounded or oblong in shape, and usually with a smooth outline. When colonies were removed and stained they were found to contain two types of organisms, one a very minute bacillus, the other an oblong form resembling *Nitrosomonas* but considerably smaller. When inoculated into bouillon, growth resulted in two or three days. The colonies on the plate varied in size from 30 to 150 μ.

After the plate had shown nitrite formation but little change in growth could be detected. The same type of colonies predominated and a few more had developed, which also were very small and very light brown in color. When isolated colonies were removed from the plate by means of the Barber apparatus and placed in the usual liquid medium, oxidation resulted in about one case out of twelve or more. When stains were made from those showing oxidation, apparently only the *Nitrosomonas* form was present, but when examined very closely the small bacillus form could be found. Bouillon was used as a test for purity since Winogradsky states that neither of the nitrifying organisms would produce visible growth in that medium. When bouillon tubes were inoculated from the culture in solution, which was secured from

the agar colony, conflicting results were secured. The bouillon tubes were inoculated with one or two loops from the active culture. Some of the tubes thus inoculated produced growth while others did not. Those giving no growth were considered pure and transfers were made to fresh media. The subcultures showed prompt oxidation and when bouillon was inoculated from them some produced growth and others did not. These results were very confusing. If the mother culture proved "bouillon sterile" why should the daughter cultures show growth in bouillon? It was then found that if 0.5 cc. of each culture was used as inoculum for the bouillon, growth resulted in every case. From the results it appears that the cultures were not pure but contained another organism which was present in such small numbers in comparison to the number of *Nitrosomonas* cells that occasional loops could be secured which contained none of the contaminating form. Working on this supposition, a great number of dilution tests were made and media inoculated from each dilution. The results showed that the cultures contained a very high number of nitrite-forming organisms, in some cases between 10 and 20 million per cubic centimeter of solution. Each flask inoculated from the respective dilutions was promptly inoculated into bouillon as soon as it had shown oxidation, 0.5 cc. of the solution being used as the inoculum. Some gave growth in bouillon while others did not. The results indicated that the former supposition is not correct, but that the contaminating form was present in larger numbers than *Nitrosomonas* or was so closely associated with it that the ordinary dilution methods would not separate them. In certain cases the mother culture would not produce growth in bouillon while the F_1 cultures would produce growth in bouillon and similar results with the F_2 generation. This peculiarity has been noted several times.

Only one, or occasionally two, types of growth would appear in the bouillon tubes inoculated from the cultures. Later in the work only a very small bacillus form was found. If the cultures contained only two contaminating forms they should be separated easily from *Nitrosomonas* by the plate method, since the latter organism is present in such great numbers. Many agar plates were inoculated from these cultures in various dilutions. Some of the plates produced colonies and prompt oxidation of the ammonium salt while others gave entirely negative results. When studied under the microscope the plates were found to contain numerous small brown colonies, the same as those previously described, and transfers from these colonies to the nutrient solution gave the same results as those secured before; that is, about 10 per cent or less gave oxidation of the ammonium salt, and these gave sporadic growth when inoculated into bouillon.

When dilutions were made from the cultures in an effort to eliminate the contaminating form, bouillon tubes as well as the ammonium sulfate nutrient solution were inoculated from each of the dilutions. In the majority of the cases the bouillon tubes remained free from growth after 2 weeks' incubation, even from the lower dilutions, while the flasks of the nutrient solutions, inoc-

ulated from the same dilutions, gave prompt oxidation and in many cases subsequent growth in bouillon.

Silicic acid gel plates. The inexplainable results secured from the use of agar as the base for a solid medium led to the use of silicic acid gel as a substitute. The method of preparing this as a medium in this work has been previously described. The results obtained were somewhat more encouraging. This medium when inoculated with an active nitrite-forming culture showed the formation of nitrite after an incubation of 3 to 4 weeks. Growth was scarcely visible without the aid of a microscope, but when examined under the 16-mm. objective numerous very small light brown colonies could be seen. These colonies did not develop as large as those on the agar but remained very small. At first they were round and regular in outline but after the addition of more ammonium sulfate to the plate and longer incubation, scattered cells could be seen around the colonies. Close examination showed that the colony had become so dense that it had bursted apart and the single cells were moving outward from the center. The colonies were very difficult to remove from the medium and extremely difficult to tear apart. If a colony was removed by the method previously described, placed on a slide and stained with carbol fuchsin, in the majority of cases no organisms could be found. Even when small chunks of the silicic acid gel containing several of the colonies were smeared on the slide and stained, no organisms could be found or if so they were in such dense clumps that single organisms could not be studied. For a while this was very confusing but after a time an explanation was found. The organisms clung tightly to the silicic acid and in the fixing they became attached to the small particles of silicon dioxide (SiO_2). This conclusion was formulated from the discovery that the colonies could be stained directly on the gel. Carbol fuchsin was poured directly on the surface of the gel, allowed to remain from 40 to 80 seconds, then washed off with a slow stream of water. If it was not desirable to stain the whole plate a small portion of the gel was removed by means of a thin glass slide and stained in the same manner. The colonies were deeply stained while the gel contained no noticeable stain. Photomicrographs of the colonies stained by this method are shown in plates 4 and 5.

The colonies of *Nitrosomonas* were not always as described above. It was very difficult to secure silicic acid gel plates of the same physical composition, and the consistency of the gel determines the nature of the colony. On the softer gels the colonies were not so compact. The colonies appeared first as small refractive bodies with sharp outline and later they became light brown in color and continued to darken until fairly dark brown. As they became older the outline became more irregular and a number appeared as those shown in plate 5. To the naked eye the colonies appeared light gray in color.

Other colonies not *Nitrosomonas* were seen on the plates inoculated from the crude cultures, which were very similar in size, color, and shape to the *Nitro-*

somonas colonies.   These non-nitrifying colonies were small, rounded or oblong in shape, and brown in color.   A few of the colonies had a granular appearance, a sharp outline within which the organisms were somewhat loosely arranged approaching a more dense granular center.   This type of colony was usually larger than the others and was found on the surface.

When colonies were selected from the gel and inoculated into the usual ammonium sulfate nutrient solution, nitrite formation resulted with great irregularity.   A small percentage produced oxidation of the ammonia, probably a slightly higher percentage than was secured from the agar colonies. Subsequent tests with bouillon gave the same results that were secured from the use of agar.   One interesting fact which had not been previously noted in the use of agar was brought out from the use of the silicic acid gel.   On several different occasions plates were secured which contained a large number of colonies and which gave an active oxidation of the ammonium salt.   These colonies answered in every detail the description of the *Nitrosomonas* colony given by Winogradsky and were identical with his photographs.   The colonies were transferred to the usual ammonium sulfate medium but produced no oxidation, and when transferred to bouillon they produced no growth.   Great numbers were then scooped from the plate and used as inoculum into each medium, with negative results.   This fact was later noted in many cases when agar plates were used.   When stained the organisms were not typical of *Nitrosomonas* as found in the active state in liquid medium but were smaller, yet they still maintained their characteristic shape.

There was on hand in the laboratory one culture, no. X, which produced active oxidation but would produce no growth when inoculated into bouillon repeatedly, yet its daughter cultures, after oxidation of the ammonia, produced growth in bouillon.   One of the members of the department wished to test the results secured with this culture.   His tests with bouillon likewise gave negative results.   He then inoculated 10 parallel flasks of fresh ammonium sulfate medium from this culture, first running controls on the medium used, and when the ammonium salt had been oxidized tested for purity by the inoculation of bouillon.   After oxidation of the ammonium sulfate more was added and the bouillon test repeated, etc., until no further oxidation could be secured.   Each of the 10 cultures produced growth in bouillon throughout the entire length of the experiment.   Fourteen additions of ammonium sulfate were made and likewise 14 bouillon tests, each of which produced growth. After no further oxidation could be secured 6 of the cultures were analyzed for nitrite content.   They contained the following amounts of nitrogen as nitrite, calculated per 100 cc. of solution culture: 1 = 184 mgm., 2 = 170 mgm., 3 = 208 mgm., 4 = 206.6 mgm., 5 = 106.6 mgm., 6 = 180 mgm.

The results secured from this experiment show that the bouillon test was not influenced by the nitrite content of the solution used as inoculum.   In all cases 0.5 cc. of the culture was used as inoculum for 10 cc. of the bouillon.

This sporadic growth in bouillon may be described best by the results of one series which are shown in figure 1. Several of such series of tests have been made and this particular one affords a representative example of the results secured in each case. This chain began with culture X which was just described. This culture oxidized each added portion of ammonium sulfate until the solution contained 218.9 mgm. of nitrogen as nitrite per 100 cc. of solution.

The sporadic growth in bouillon in the various generations is evident from a glance at the chart. All the daughter cultures were not made on the same date but at different times; however the same medium was used throughout. It was thought that this periodic growth in bouillon could be explained by a loss of virulence of the nitrite-forming organism, since some of the cultures in the chart failed to produce oxidation of the ammonium salt. Perhaps the organism would produce growth in bouillon when possessing its usual activity, but when a weakness in its oxidizing power began to express itself the bouillion test would give negative results. This possibility has not been sustained by the results with a majority of the cultures. Some cultures showing unusual activity produced no growth in bouillon. However, those cultures showing low oxidizing ability in general give negative results when inoculated into bouillon. Bouillon of varying degrees of acidity and alkalinity was used and the results indicated that the neutral bouillon was equally as satisfactory as bouillon slightly acid or slightly alkaline. *Many transfers have been made from the growth in the bouillon tubes to fresh ammonium sulfate medium but in no case has oxidation resulted.* The growth secured in the bouillon was quite characteristic in all cases—first very scanty and requiring an incubation of 6 to 10 days, then giving the medium only a very slight cloudiness. It was very difficult to secure a characteristic stained preparation from the bouillon, but in general the organism was very small, less than 1 μ in length, a distinct bacillus, often appearing as a mere dot.

No explanation can be given for the fact that a few of the cultures shown in the chart failed to oxidize the ammonium salt. This failure to oxidize was not met with in the cultivation of the crude cultures but was noticeable in all the pure cultures. This indicates that the true nitrite-forming organism is aided by the contaminating forms present in the enrichment cultures.

From the results obtained in the foregoing work it is obvious that bouillon cannot be used as a test for purity of the nitrite-forming organism unless other confirmatory tests be made, such as cultivation on agar or silicic acid gel.

Winogradsky found the nitrite-forming organism in his cultures in two stages, one of which he terms "schwärmer" and the other the "free cell stage." It is doubtful if such forms were present in this work, though such might have been the case. The free-cell type was common in all the cultures, and many strains showed great masses of the organisms which resembled very closely the "schwärmer" stage; however, this latter character of growth differed but slightly from the forms found as free cells and was not considered to be a mor-

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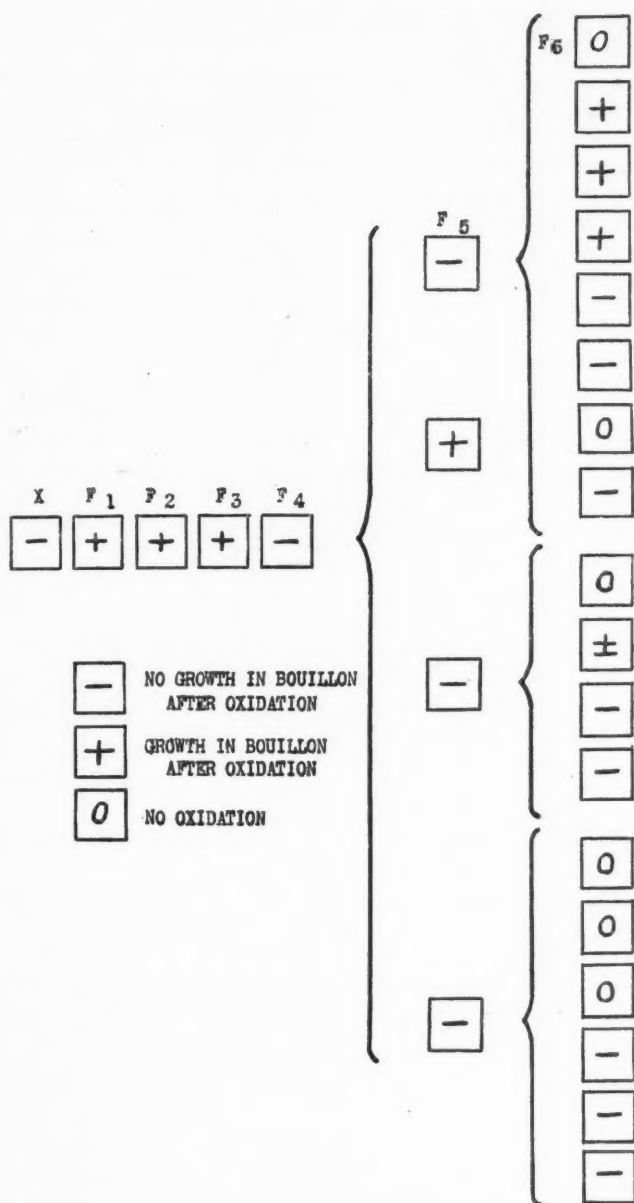
Fig. 1. CHART SHOWING RESULTS OF BOUILLON TESTS FROM NITROSOMONAS CULTURES

phologically different form, although the organisms within the group were occasionally slightly smaller.

When washed agar plates were prepared and inoculated from bouillon sterile cultures active growth and oxidation resulted in many cases. Colonies developed in very great numbers but very few of these would produce oxidation when transferred to the ammonium sulfate nutrient solution, and none would produce growth when transferred to bouillon. This inability of the organism to oxidize the ammonium salt in solution when transferred from the plates was a very puzzling problem. It was thought possible that there was a symbiotic relationship between the type growing in bouillon, if a contaminating form, and the true nitrite-forming organism. If this was the case each would lend its influence on the agar plate and when one of the forms was transferred to liquid medium oxidation would not result because of the absence of the other form. In order to check this possibility many colonies were selected from agar and silicic acid gel and transferred to flasks of the nutrient solution. These flasks were then inoculated from the characteristic bouillon growth as well as with colonies selected from Heyden agar plates which had been inoculated from a crude culture. The results in general were negative, indicating that if such symbiotic relationship existed it was destroyed by the development of the organisms on agar. It is not believed that any organism other than *Nitrosomonas* was present on the plates. The fact that *Nitrosomonas* colonies develop on washed agar and silicic acid gel, and maintain their oxidizing power on such plates, and then produce oxidation in such a small percentage of cases when transferred from the plates to ammonium sulfate nutrient solution, is explained in that the conditions met with on the plates are not entirely favorable and have brought about a weakening of the organism which is first detected when transfers are made to the original liquid medium. This necessitates the use of a large inoculum, which explains the failure in many cases to secure oxidation from a single colony.

It was thought for a long time that this growth in bouillon was the nitrite-forming organism but later results proved that such was not the case. Pure cultures have been obtained from colonies and held on hand for many generations without producing growth in bouillon. The sporadic appearance of the growth in bouillon in the early work is held to be due to contamination. This small bacillus is so closely associated with the nitrite-forming organism and is present in such small numbers that it is not consistent in showing its presence. The nutrient requirements of the organism are so slight that it is able to develop slightly in the nitrifying solution with the nitrite-forming organism.

Accumulation of nitrite in liquid cultures. The extremely high concentration of nitrite which could be withstood by the organism was noticeable throughout the work. The nitrite content of one series of cultures has already been given, one of which contained 208.1 mgm. of nitrogen as nitrite per 100 cc. of solution. Another experiment was planned to determine the concentration of nitrite which would be formed before the activity of the organism was entirely pro-

hibited.   Two 1-liter Erlenmeyer flasks, each containing 100 cc. of the am-
monium sulfate nutrient medium, were inoculated from an active culture and
incubated at 28°C.   As soon as the cultures showed the absence of ammonia
by chemical test 1 cc. of sterile 10 per cent ammonium sulfate solution was
added to each, and this continued until no further oxidation of the ammonium
salt could be secured.   Culture I always gave negative results when inoculated
into bouillon, while its duplicate II always produced growth.   Sub-cultures
from each gave prompt oxidation of the ammonia and subsequent tests with
bouillon gave the usual periodic growth outlined in previous discussion.   The
flasks contained magnesium carbonate in excess, so no visible growth could be
seen in either.   After a long period of incubation the magnesium carbonate
became exhausted from the formation of nitrous acid and the solutions became
clear.   It was then noted that a flaky material was forming in culture I while
culture II was almost entirely free from it.   Sterile magnesium carbonate was
then added to each culture and the additions of ammonium sulfate continued.
The flaky material continued to develop in culture I and soon appeared in
culture II.   Stain preparations from this material showed it to consist of
large masses of the *Nitrosomonas* cells, disintegrated material, and a few crys-
tals of some insoluble salt.   After no further oxidation could be secured stains
of this material showed it to contain relatively few typical *Nitrosomonas* cells,
but large numbers of disintegrating cells.   Evidently the high concentration
of nitrite was bringing about the destruction of the organism.   The cultures
were then analyzed for nitrite content and culture I found to contain 218.9
mgm. of nitrogen as nitrite and culture II 201 mgm. of nitrogen as nitrite per
100 cc. of solution.

Thermal death-point.   The thermal death-point was determined by use of
actively oxidizing pure cultures in liquid media.   Tubes containing 10 cc. of
0.7 per cent sodium chloride solution were placed in the water baths at vari-
ous temperatures and held until of uniform temperature throughout.   Then 0.5
cc. of the culture was pipetted into each tube, the contents thoroughly shaken,
and held at the temperature for 10 minutes; 1 cc. was then drawn from the tube
and used as inoculum in the usual ammonium sulfate nutrient solution.   All
tests were run in duplicate at different times, different cultures being used.
The thermal death-point was found to be between 53 and 55°C.

Longevity.   The question of preserving cultures in the laboratory for rela-
tively short periods of time is one which causes no anxiety.   Active cultures
in solution were capped with tinfoil and set aside in lockers in the laboratory,
and after the elapse of two months the organisms were able to resume activity
immediately on introduction into fresh medium or on the addition of more
ammonium sulfate solution.

Test tubes containing soil were sterilized in the autoclave and after being
allowed to aerate several days were moistened with very dilute ammonium sul-
fate solution and inoculated with the organisms.   Some of the tubes were
sealed with the soil in moist condition while others were left with cotton plugs.

The organisms were found to be present after 10 months' incubation at room temperature, both in the sealed tubes and those in which the soil had become air-dry, and able to bring about the oxidation of the ammonia when brought into the nutrient solution.

Field soil which had been air-dried and passed through a 100-mesh sieve and stored in tightly stoppered bottles was found to contain the organisms after the lapse of nearly 7 years.

Sensitivity to light. Cultures of both *Nitrosomonas* and *Nitrobacter* are very sensitive to light. When incubated in the laboratory neither organism will produce oxidation unless protected from the light. Direct sunlight causes complete destruction of the organism, while diffused light merely prevents their activity unless exposed for a long period of time, which proves fatal.

Contaminating forms. There are three forms which remain present in the enrichment cultures of both *Nitrosomonas* and *Nitrobacter*. On washed agar one type forms a very small light colored colony, visible to the naked eye, which is also nearly colorless when examined under the microscope. The colony has a characteristic thin and dull appearance. The other type appears to the naked eye as a very small compact yellowish brown dot, and is dark yellow or brown when examined under the microscope. Both types produce growth in bouillon. Both develop sparingly on slopes of bouillon agar, Heyden agar, and mannite agar, the latter form producing a yellow pigment on the Heyden agar. The pigment-forming organism brings about a reduction in nitrate broth with the formation of nitrite and ammonia. The other organism has no reducing action. No growth was secured when the following media were inoculated with each organism—milk, gelatin, Giltay's solution, sucrose broth, lactose broth, and urea broth.

Both organisms are stained fairly well with carbol fuchsin. The yellow-pigment former is a very small bacillus ablut 0.4μ wide and 1μ long, always single and never found in chains. The other is a very small coccus, or sometimes slightly oval, about 1μ in length, often found in pairs. Both organisms are gram-negative. These two forms were eliminated before the unusual results with bouillon above recorded were secured. They both produce much heavier growth in bouillon than the type of growth observed in the bouillon-sterile series of cultures, and only a small amount of inoculum is necessary to produce the growth in bouillon.

The third contaminating form is the one found in comparatively pure cultures and the one which has given so much trouble throughout this work. It produces colonies on washed agar and silicic acid gel very similar in gross appearance to the nitrifying organisms. It produces very slight turbidity in bouillon after an incubation of several days. It is a minute bacillus easily stained with carbol fuchsin. It is hoped at a later time to give a more complete report on the morphological and physiological characteristics of this organism.

SUMMARY

The enrichment process with cultures of both *Nitrosomonas* and *Nitrobacter* was continued for a long period of time. The results with both series of cultures plainly indicated that the enrichment process could be continued for an indefinite period of time without the slightest loss of activity of the organisms. Experiments with these cultures were made to determine the number of nitrifying bacteria which they contained, the number of contaminating organisms, and the effect of various treatments on the oxidizing power of the organisms. The number of nitrifying bacteria increased from relatively few to more than 10 million per cubic centimeter of the liquid medium. Further tests by dilution and plate methods showed that foreign organisms were present in the cultures in greater numbers than the nitrifying organisms, consequently a pure culture could not be obtained by the enrichment process alone. It was found that silicic acid had no effect on the activity of the organisms. Soil extract was also found not toxic. When the extract was used to prepare the nutrient solutions the activity of the organisms was equally as great as when conductivity water was used. *Nitrosomonas* cultures withstood a concentration of 1.00 per cent sodium chloride but oxidation was greatly retarded at that concentration.

Plates and slopes of washed agar and silicic acid gel were used with success throughout the work. The gypsum block did not give satisfactory results; so its use was discontinued early in the work. The colonies which developed on either washed agar or silicic acid gel were very small, which condition necessitated the use of the microscope in all colony study. Single colonies were removed from the plates by means of a modification of the Barber apparatus. These colonies were transferred to liquid media and their activity measured by the rate of oxidation of the nitrogen salt. All colonies of *Nitrobacter* thus transferred to liquid media did not produce oxidation of the nitrite; this did not signify that the particular colony was not *Nitrobacter*, but is explained in that the organism is somewhat weakened by the growth on the solid medium, and a sufficient quantity of inoculum is not secured from the single colony. *Nitrobacter* cultures were tested for purity by inoculating into bouillon, with 0.5 cc. as inoculum. Pure cultures would produce no visible growth in this medium.

Colonies of *Nitrosomonas* transferred from plates to liquid medium usually failed to produce oxidation. This was first thought to be due to contaminating forms but this conclusion was later proven to be erroneous. When plates were inoculated from pure cultures of *Nitrosomonas* only 10 to 20 per cent of the colonies which developed would produce oxidation when transferred to liquid medium. This was due to the fact that the organism was weakened by its development on the solid medium and sufficient inoculum was not used when transferring back to the liquid medium. Those producing oxidation when introduced into the liquid medium represented those colonies in

which the organisms were not materially weakened by the conditions in the solid medium.

The bouillon test with *Nitrosomonas* was at first very confusing. The results outlined in previous pages indicate that this organism will not produce growth in bouillon. When growth was secured in bouillon inoculated from cultures of *Nitrosomonas* they were considered impure. In a careful review of the literature on the isolation of *Nitrosomonas* it is found that many investigators observed this bouillon form in their pure cultures, and some conclude that it is the true *Nitrosomonas*, which is pleomorphic. Many investigators report the presence of this bouillon form in their cultures and their work has been given more or less skeptical criticism because of such growth. The work of Stutzer and Hartleb, in regard to the organism *Hyphomicrobium*, introduces another factor which, to the present time, has not been completely worked out.

CONCLUSIONS

Pure cultures of *Nitrosomonas* and *Nitrobacter* were isolated from soil and cultivated on artificial media.

Both *Nitrosomonas* and *Nitrobacter* develop readily on plates of washed agar or silicic acid gel. The latter medium is more satisfactory in the work of isolation, disregarding its difficulty of preparation, but after pure cultures are secured washed agar can be used with success.

The colonies which developed on the plates were extremely small and required the use of the microscope in the study of their characteristics. Isolated colonies were removed from the medium by means of a modification of the Barber apparatus.

Pure cultures of either *Nitrosomonas* or *Nitrobacter* will produce no visible growth when inoculated into bouillon. In using bouillon as a purity test 0.5 cc. of the culture must be used as inoculum to give reliable results.

Pure cultures of these organisms can be maintained in liquid medium for an indefinite period of time.

The enrichment process with both *Nitrosomonas* and *Nitrobacter* can be continued for an indefinite period of time without the slightest loss of activity of the organisms. The F_{62} enrichment showed as great activity as any of the preceding generations.

Neither the enrichment process nor the securing of "super-enrichment" cultures will yield a pure culture of *Nitrosomonas* or *Nitrobacter* without the use of suitable solid media.

By careful manipulation the number of nitrifying organisms in the enrichment cultures can be increased from relatively few to a number greater than 10 million per cubic centimeter of the culture solution.

Soil extract used to prepare the nutrient solutions for the cultivation of both *Nitrosomonas* and *Nitrobacter* did not prove toxic in either case.

Sodium chloride in a concentration of 1.00 per cent was very toxic toward *Nitrosomonas*.

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PLATE 1

COLONIES OF NITROBACTER; DEEP-SEATED COLONIES ON WASHED AGAR; UNSTAINED;
MAGNIFICATION 350 DIAMETERS

PLATE 2

NITROBACTER FROM CULTURE IN LIQUID MEDIUM; STAINED WITH CARBOL FUCHSIN; $\times$ 1540

475
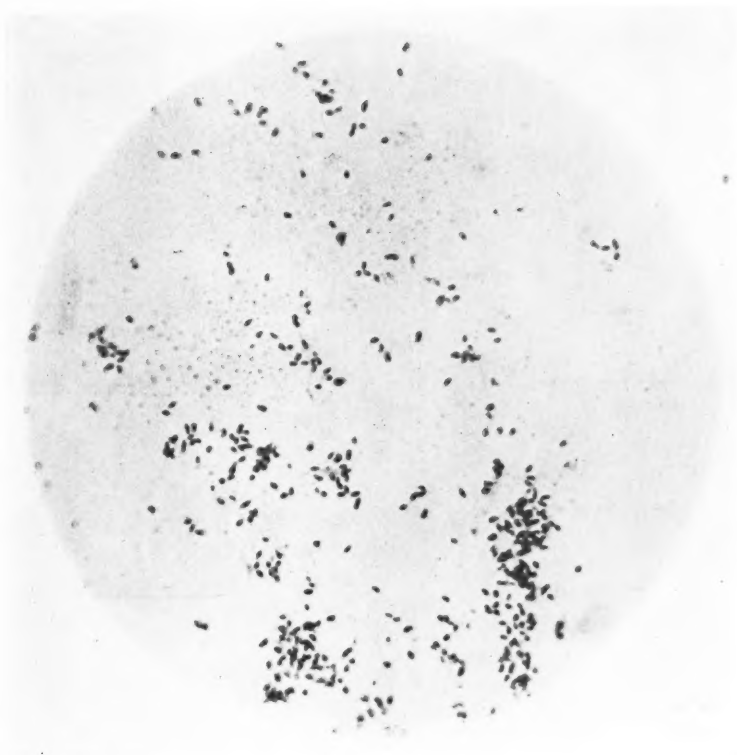
PLATE 3

NITROBACTER FROM CULTURE IN LIQUID MEDIUM; STAINED WITH CARBOL FUCHSIN; $\times$ 2500

476
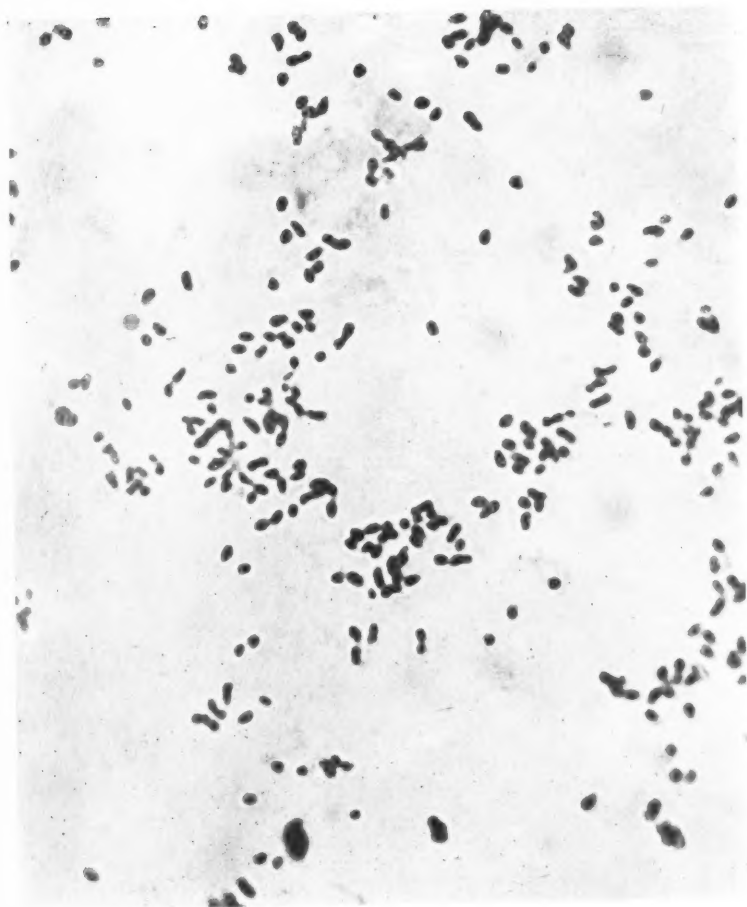
PLATE 4

SURFACE COLONIES OF NITROSOMONAS ON SILICIC ACID GEL; STAINED WITH CARBOL FUCHSIN
WITHOUT REMOVING FROM THE GEL; × 200

PLATE 5

Surface Colony of Nitrosomonas on Silicic Acid Gel; Stained with Carbol Fuchsin
Without Removing from the Gel; × 1200
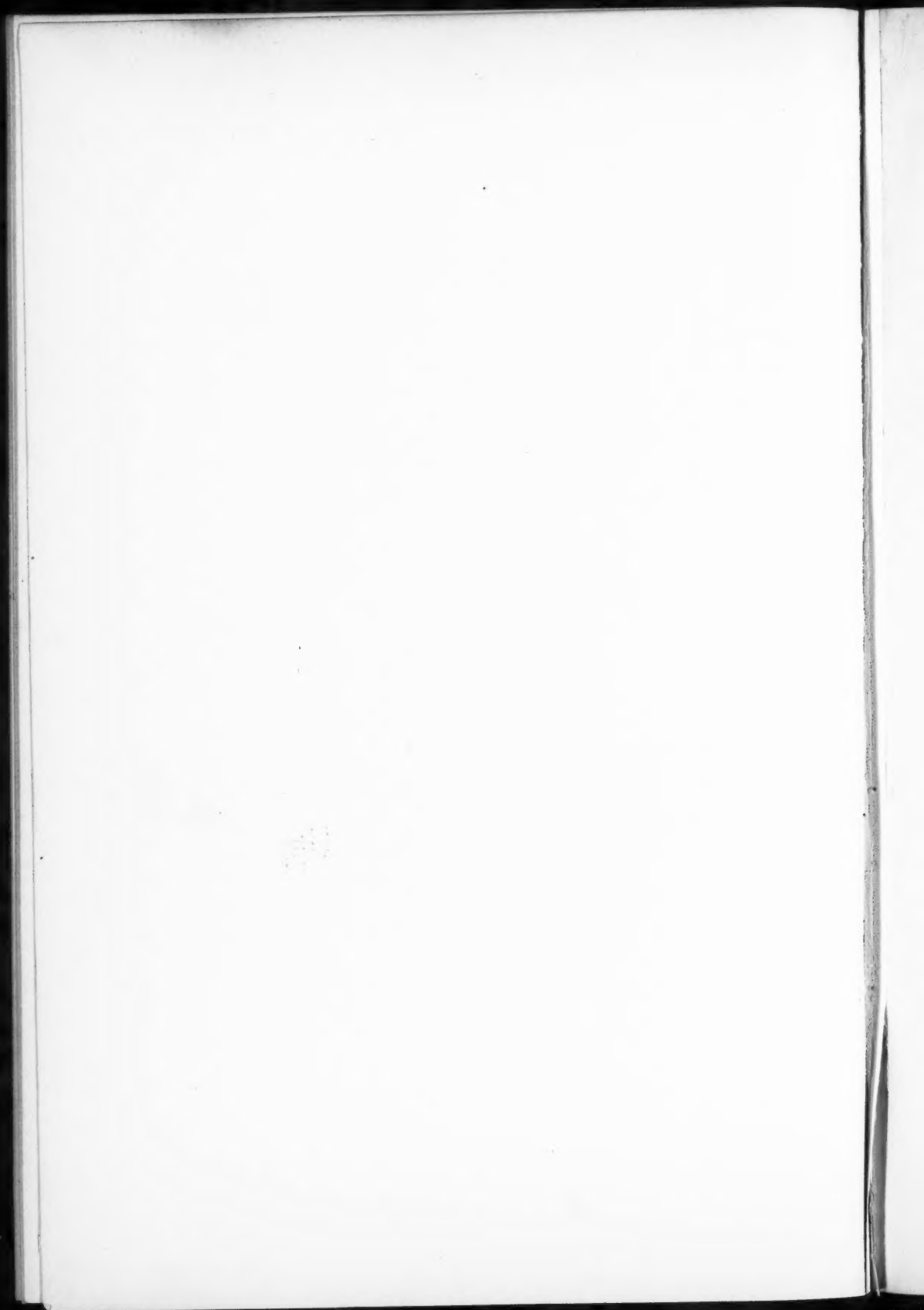
THE EFFECT OF CERTAIN NITROGENOUS AND PHOSPHATIC FERTILIZERS ON THE YIELD OF CRANBERRIES

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The study of fertilizers for cranberries is being carried on by the experiment stations of Massachusetts, Wisconsin and New Jersey.

The Massachusetts Agricultural Experiment Station has been conducting fertilizer experiments for ten years. Dr. Franklin (3) of that station finds under Cape Cod conditions, that although plots treated with commercial fertilizer give a slightly larger crop, the increases are not sufficient to pay for the fertilizer, and the labor expended in applying it.

The Wisconsin Agricultural Experiment Station (4), on the other hand, recommends the use of commercial fertilizers on Wisconsin bogs. It suggests the following applications per acre:

	pounds
Acid phosphate..	240
Nitrate of soda..	80
Sulfate of potash..	80

The New Jersey Agricultural Experiment Station has been investigating the use of plant-food for cranberries in a general way since 1913 (1, 2, 5, 6, 7, 8, 9, 10). The preliminary work showed that there were three types of cranberry soil: savannah, mud and mud underlaid with iron ore deposits. The savannah soil was a sand with enough dark silt to give the whole a black appearance; the growers often call it "hard-bottom." Mud, on the other hand, consists of from 10 inches to 20 feet of peat usually over hardpan. Mud sometimes occurs on top of iron ore deposits and in such cases its composition differs from that on ordinary hardpan.

The plant-food work of the New Jersey station between 1913 and 1918 was limited to testing the effect of various plant-foods and the best sources of each ingredient. The results brought out the following six points:

(1) Both nitrogen and phosphoric acid either alone or combined gave good results on savannah soil.

(2) Sulfate of ammonia alone was an undesirable source of nitrogen.

(3) Applications of phosphoric acid gave good results on mud and mud underlaid with iron deposits.

(4) Nitrogen derived from nitrate of soda gave results easily shown in quickened vine growth during the week following the application while nitrogen

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from dried blood did not show its effect for from 3 to 5 weeks after application. The nitrogen in either case had some effect on the crop during the year in which it was applied.

(5) Phosphoric acid derived from acid phosphate was immediately available while phosphoric acid derived from rock phosphate was not available until the year following its application, but when it became available it was as desirable as phosphoric acid from acid phosphate.

(6) Annual applications of 40 pounds of nitrogen to the acre resulted in too much vine growth.

As stated above these investigations were planned to indicate the kind of plant-food needed on the cranberry bog and the desirable sources of such plant-food.   During 1919 studies were started to determine the quantity of the beneficial plant-foods desirable for annual application; as well as to test the more promising new sources of plant-food.   The studies were undertaken to determine the following:

(1) The optimum amount of nitrogen which should be applied annually to savannah soil.

(2) The derivation of needed nitrogen for savannah soil from a combination of mineral and organic sources.

(3) The optimum amount of phosphoric acid for savannah, mud and iron ore bottoms.

(4) The optimum amount of a tentative formula for mixed fertilizer to be applied to savannah soil.   The tentative formula was made up by the station on a basis of the results of the last six years.

(5) The value of calcium cyanamide as a source of nitrogen for savannah bottom and the value of barium sulfate on cranberry soil.

1. THE OPTIMUM AMOUNT OF NITROGEN WHICH SHOULD BE APPLIED ANNUALLY TO SAVANNAH SOIL.

2. THE DERIVATION OF NEEDED NITROGEN FOR SÁVANNAH SOIL FROM A COMBINATION OF MINERAL AND ORGANIC SOURCES

The object of this set of experiments is twofold: first, to determine the optimum amount of nitrogen which should be applied annually to savannah soil, and second, to compare the effect of nitrogen derived from mineral sources with that from organic sources.

Former studies conducted by this station have shown that 40 pounds of nitrogen is undoubtedly too much to apply to an acre of cranberries annually; also, in the experience of one of the growers, 10 pounds had been applied without beneficial results.   Obviously, the optimum was somewhere between these amounts and the station determined to try 20 pounds and 30 pounds.

The search for the best source of needed nitrogen, from the experience of the station with a variety of substances, narrowed itself down to nitrate of soda and dried blood.   Either of these substances becomes readily available

on being applied to cranberry soil, the nitrate of soda almost immediately and the dried blood in two or three weeks.   It was noted that when an application of nitrogen was made entirely from nitrate of soda, the vines made a quick start but it was assumed that the plants were unable to maintain a satisfactory rate of growth during the season, and the formation of fruit buds for the following season was reduced.   Dried blood in comparison with nitrate of soda started slowly and at the end of a month was most active.   This suggested the question whether or not a mixture of nitrogen from the two sources might be desirable and, accordingly, a test was made with results as given in table 1.

TABLE 1

Results of nitrogen experiments on savannah soil; variety, Early Black

PLOT	TREATMENT PER ACRE	NITROGEN	YIELD	INCREASE OVER CHECKS
			lbs. per acre	*per cent*
F-SB-N 1	Nothing...............................		3320	
F-SB-N 2	140 pounds sodium nitrate................	20	4280	37
F-SB-N 3	Nothing...............................		2920	
F-SB-N 4	70 pounds sodium nitrate; 85 pounds dried blood.................................	20	4400	48
F-SB-N 5	Nothing...............................		3000	
F-SB-N 6	170 pounds dried blood....................	20	3200	−2
F-SB-N 7	Nothing...............................		3560	
F-SB-N 8	210 pounds sodium nitrate................	30	7320	91
F-SB-N 9	Nothing...............................		4100	
F-SB-N 10	105 pounds sodium nitrate; 127½ pounds dried blood............................	30	4660	21
F-SB-N 11	Nothing...............................		3160	
F-SB-N 12	255 pounds dried blood....................	30	3920	10
F-SB-N 13	Nothing...............................		3900	

This study was made on land as nearly uniform as was available.   Our former experience had shown that uniform vine growth did not indicate uniform ability to produce a crop.   In order to obtain results as accurate as possible, each treated plot was placed between two check plots, and the crop of the treated plot compared with the average of the checks.

As was expected, Plot F-SB-N-2 and Plot F-SB-N-8 started quickly with a dark green foliage.   F-SB-N-4 and F-SB-N-10 showed quickened vine growth somewhat later, and in about six weeks F-SB-N-6 and F-SB-N-12 showed more vine growth than the checks.   The results indicate that an application of 210 pounds of sodium nitrate to thin vines on savannah land will tend to produce a heavy covering of vines and an excellent crop.   The mixture of nitrate of soda and dried blood was not as good as pure nitrate of soda within the first year.   This investigation should produce some interesting results in the second year.

3. THE OPTIMUM AMOUNT OF PHOSPHORIC ACID FOR SAVANNAH, MUD AND FROM ORE BOTTOM

It is recognized that a sufficient amount of phosphoric acid is lacking in most cranberry soils, but the amount that may be applied economically each year is not definitely known. The study here reported was designed to throw light on this problem.

In former investigations conducted by this station phosphoric acid derived from acid phosphate gave immediate returns; on the other hand, phosphoric

TABLE 2
Results of phosphate tests; variety, Early Black

PLOT	TREATMENT	P_2O_5	Savannah SB-F-P		Mud MB-F-P		Iron ore IB-F-P	
			Pounds per acre	Per cent gain	Pounds per acre	Per cent gain	Pounds per acre	Per cent gain
	lls. per acre	*lbs.*						
1	Nothing..........................		2440		7600		2340	
2	125 pounds acid phosphate..............	20	2280	−3	9660	18	2000	−10
	75 pounds phosphate rock..............	20						
3	Nothing..........................		2240		8700		2080	
4	250 pounds acid phosphate..............	40						
	150 pounds phosphate rock..............	40	3720	29	9000	5	3160	90
5	Nothing..........................		3520		8060		1160	
6	375 pounds acid phosphate..............	60						
	225 pounds phosphate rock..............	60	3200	−4	10140	20	1180	2
7	Nothing..........................		3120		8820		960	
8	500 pounds acid phosphate..............	80						
	300 pounds phosphate rock..............	80	3580	17	11320	16	1840	30
9	Nothing..........................		3000		10900		1880	
10	150 pounds phosphate rock..............	40	3000	2	9160	16	2320	0
11	Nothing..........................		2880		10820		2840	
12	150 pounds soft phosphate rock..........	40	2840	−2	8340	−9	3820	6
13	Nothing..........................		2920		7640		4400	
14	250 pounds acid phosphate..............	40	3440	11	8720	18	4460	37
15	Nothing..........................		3360		7340		2260	

acid from rock phosphate gave only moderate increases until after the first year, but when it began to operate in any marked degree its results were quite as good as those from acid phosphate. Rock phosphate, because of its alkaline reaction, did not leave an undesirable residue in the soil and it was the most beneficial of the materials already tested. This was used as the basis of the treatments. As rock phosphate would not become effective until the second year, a treatment was made of an equal amount of phosphoric acid derived from acid phosphate. This extra treatment was to furnish phosphoric acid

for the first year, the treatment in following years, except on plot 4, to be merely rock phosphate. The treatments and yields are given in table 2.

Series IB-F-P was located on low ground where usually there is sufficient drainage but during the current year a large amount of rainfall kept the soil saturated and the results are not the best that could be expected.

The test shows that 300 pounds of rock phosphate is not enough to cause damage. Applications of acid phosphate caused increases in yield rather consistently but rock phosphate was not active in the first year. These figures of course, are based on the one year's crop, and the crop of the second year is expected to show more definite results.

4. THE OPTIMUM AMOUNT OF A TENTATIVE FORMULA FOR MIXED FERTILIZER TO BE APPLIED TO SAVANNAH SOIL

The station felt that it was ready to publish a tentative formula for a complete fertilizer for savannah land and determine as nearly as possible the amount needed for annual applications. It was made up on the basis of the last six years' results and is as follows:

	pounds
Sodium nitrate	75
Dried blood	75
Rock phosphate	300
Sulfate of potash	50

The first year this material was used 300 pounds of acid phosphate was added in order to have phosphate available the year of application.

The results of tests with this mixture are given in table 3.

TABLE 3

Results of tests with a mixed fertilizer on savannah soil; variety, Early Black

PLOT	TREATMENT	YIELD	INCREASE OVER CHECKS
	lbs. per acre	*lbs. per acre*	*per cent*
SB-F-C-1	Nothing	3800	
SB-F-C-2	264 pounds mixture; 176 pounds acid phosphate	4780	20
SB-F-C-3	Nothing	4000	
SB-F-C-4	528 pounds mixture, 352 pounds acid phosphate	5180	20
SB-F-C-5	Nothing	4680	
SB-F-C-6	792 pounds mixture, 528 pounds acid phosphate	6340	38
SB-F-C-7	Nothing	4500	
SB-F-C-8	1056 pounds mixture, 704 pounds acid phosphate	5200	49
SB-F-C-9	Nothing	2860	

Plot SB-F-C-9 proved abnormally poor and on this account Plot SB-F-C-8 showed a high percentage of gain, although it actually yielded a smaller crop than Plot SB-F-C-6.

That Plot SB-F-C-8 was over-fertilized was shown by the excessive vine growth on the plot and the many "runners" that appeared on top of the vines. The crop was somewhat less than on SB-F-C-6 which was in excellent condition. Plots 2 and 4 are expected to give much better yields the second year.

5. THE VALUE OF CALCIUM CYANAMIDE AS A SOURCE OF NITROGEN FOR SAVANNAH BOTTOM AND THE VALUE OF BARIUM SULFATE ON CRANBERRY SOIL

Calcium cyanamide was tested as a cranberry fertilizer in 1919. The material used tested 18.16 per cent nitrogen. The treatments were made on savannah soil on Howe berries, with the results reported in table 4.

TABLE 4

Results of tests with calcium cyanamide and barium sulfate on savannah soil; variety, Late Howe

PLOT	TREATMENT	YIELD	INCREASE OVER CHECKS
	lbs. per acre	*lbs. per acre*	*per cent*
1	Nothing..	5860	
2	120 pounds calcium cyanamide.............................	4900	−17
3	Nothing..	5900	
4	120 pounds calcium cyanamide, 250 pounds acid phosphate, 220 pounds sulfate of potash.............................	6000	2
5	Nothing..	5900	
6	120 pounds calcium cyanamide, 2000 pounds ground limestone.	4740	−20
7	Nothing..	5900	
8	120 pounds calcium cyanamide, 250 pounds acid phosphate, 220 pounds sulfate of potash, 2000 pounds ground limestone	6340	6
9	Nothing..	6040	

The results indicate that as a source of nitrogen calcium cyanamide gives unsatisfactory results in the first year. This is shown both when it is applied alone and when applied with limestone. The loss was not serious when used in a complete fertilizer but the gain over the checks is small.

6. TEST OF BARIUM PHOSPHATE AS A SOURCE OF PHOSPHORIC ACID

Barium phosphate was suggested as a source of phosphoric acid and was tested during 1919. The treatments and the results are given in table 5.

These results indicate that barium phosphate has little plant-food value for cranberries within the first year after its application.

TABLE 5

Results of tests with barium phosphate; variety, Early Black

PLOT	TREATMENT	P$_2$O$_5$	Savannah Pounds per acre	Savannah Per cent gain	Mud Pounds per acre	Mud Per cent gain	Iron ore Pounds per acre	Iron ore Per cent gain
		lbs.						
	lbs. per acre							
1	Nothing.............................		2920		7640		4400	
2	250 pounds acid phosphate..............	40	3440	11	8720	18	4460	37
3	Nothing.............................		3360		7340		2260	
4	150 pounds phosphate rock and 7 per cent barium sulfide........................	40	3560	8	7840	1	1060	40
5	Nothing.............................		3160		7920		1480	
6	150 pounds soft phosphate rock and 7 per cent barium sulfide...................	40	3240	9	7120	-2	2020	20
7	Nothing.............................		2760		6580		2000	
8	150 pounds barium phosphate...........	40	2400	-9	6440	-3	2940	-2
9	Nothing.............................		2480		6680		5880	

CONCLUSIONS

The experience of the first six years of cranberry plant-food studies has shown that general conclusions cannot be based upon the results gained with one year's crop, but the results are valuable in that they give some indication of what to expect in a general way. The following are the chief points brought out by the investigations:

1. Thirty pounds of nitrogen to the acre gave a better yield than 20 pounds per acre in the first year of the application. The vines receiving either amount were left in excellent condition.

2. Applications of a mixture of mineral and organic sources of nitrogen did not give a better crop than nitrate of soda alone during the first year.

3. The optimum amount of phosphoric acid to be applied was at least 80 pounds on savannah soil, mud bottom and iron ore bottom.

4. The optimum amount of the tentative mixed fertilizer for savannah bottom is 800 pounds, together with 500 pounds of acid phosphate in the first year. This amount of fertilizer, when applied to reasonably vigorous vines, helps to establish a strong growth and to increase the crop.

5. Calcium cyanamide is an unsatisfactory source of nitrogen, in the first year.

6. Barium phosphate is an unsatisfactory source of phosphoric acid in the first year.

ACKNOWLEDGMENT

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